GENETIC AND ENVIRONMENTAL INFLUENCES ON EARLY GROWTH
THE GENERATION R STUDY

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Genetic and Environmental Influences on Early Growth
The Generation R Study
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Mook-Kanamori DO, Steegers EA, Aulchenko YS, Raat H, Hofman A, Eilers PH, Jaddoe VW. Heritability estimates of body size in fetal life and early childhood. The Generation R Study. *Submitted*

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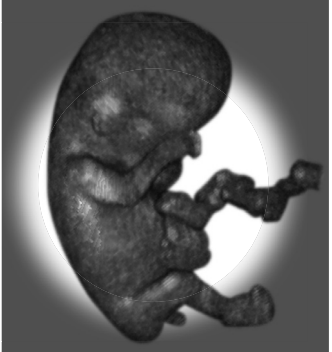
* Authors contributed equally to this work.



CHAPTER 1

Introduction

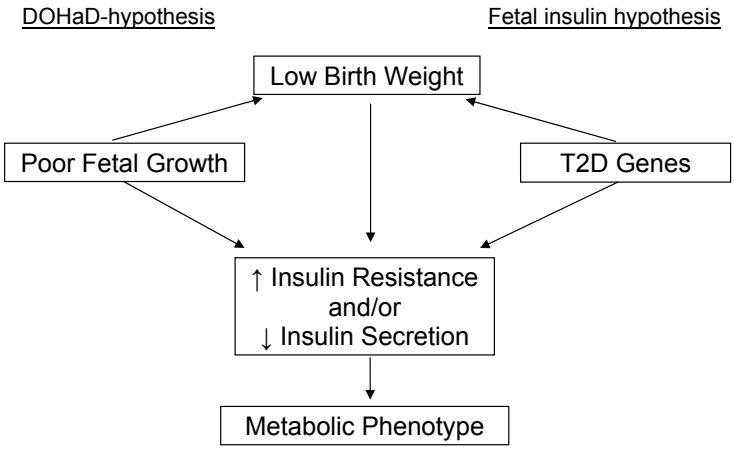
Background

Epidemiological studies have demonstrated an inverse relationship between birth weight and the risk of adult disease, such as type 2 diabetes (T2D) and cardiovascular disease.[1] These findings have led to the developmental origins of health and disease hypothesis or 'DOHaD-hypothesis'. Initially, publications reporting these associations were received with some skepticism.[2] Recently, however, these relationships have been found to be quite robust, though the effect size might not be as large as originally estimated.[3]

The main proposed causal pathway underlying the association between low birth weight and metabolic phenotype is a suboptimal fetal environment which leads to fetal undernutrition (Figure 1).[4,5] This undernutrition subsequently causes developmental adaptations that permanently alter fetal growth, physiology and metabolism, also referred to as fetal programming.[4] Though this programming might lead to an increased survival rate in early life, the developmental adaptations can have long-lasting effects on disease in adulthood.[4]

An alternative pathway for the inverse association between birth weight and type 2 diabetes referred to as the 'fetal insulin hypothesis' was proposed by Hattersley and Tooke (Figure 1).[6] They hypothesized that common genetic variants related to type 2 diabetes might also explain, at least in part, the association between poor fetal growth and metabolic phenotype in adulthood.[6] This pathway focuses primarily on the risk of type 2 diabetes in adulthood and not on other metabolic and cardiovascular disease. Since insulin is the most important fetal growth factor, these genetic variants could cause both low insulin-mediated fetal growth and low insulin secretion.[6] Recent large genetic studies on type 2 diabetes have demonstrated that insulin secretion, i.e. beta

Figure 1: Proposed causal pathways that could explain the association between low birth weight and metabolic phenotype in adulthood.



cell activity, might be much more important in the pathogenesis of type 2 diabetes than previously thought, and possibly more relevant than insulin resistance.[7]

For both causal pathways, which might be not mutually exclusive, it is important to recognize that birth weight is merely the end-point of fetal growth and the starting point of postnatal growth. Historically, most epidemiological studies that focused on the DOHaD-hypothesis used birth weight, a cross-sectional observation, as a measure of early growth.[3] However, fetal adaptations in growth and metabolism might already occur in the first weeks of pregnancy. Also, recent studies evaluating growth longitudinally have demonstrated that not only growth, but also growth rate, might also play a role in metabolic changes leading to disease in adulthood.[1] Using directly measured fetal growth characteristics from early pregnancy onwards as measure of early development may give greater insight into the timing of changes in early life that lead to the association between low birth weight and later disease.

Objectives

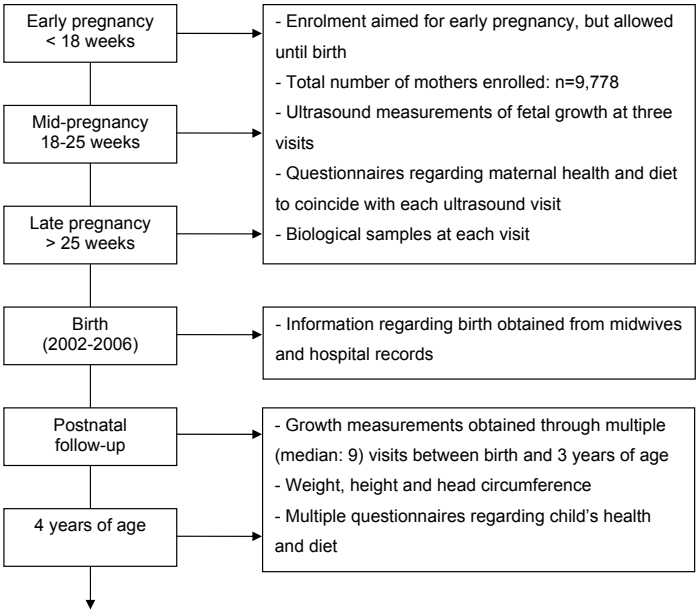
There were three main objectives of this thesis:

1. To investigate what factors influence early growth and to study how early growth influences body composition in childhood.
2. To examine the associations of genetic variants known to be associated with obesity and type 2 diabetes with growth and body composition in early childhood and adolescence.
3. To identify, by means of genome-wide association studies, new genetic variants that are associated with fetal growth.

General Design

All studies in this thesis were embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. This study is designed to identify early environmental and genetic determinants of growth, development and health.[8,9] All pregnant women living in the study area with a delivery date between April 2002 and January 2006 were eligible for enrolment into the study. While the aim was to have participants enroll during pregnancy, enrolment was allowed until the birth of the child. Detailed measurements were planned in early pregnancy (<18 weeks of gestation), mid-pregnancy (18-25 weeks of gestation), and late pregnancy (>25 weeks of gestation) and were performed using ultrasound, physical examinations, biological samples and questionnaire (Figure 2). In total, 9,778 women were included, of whom

Figure 2: Design and data collection in the Generation R Study.



8,880 were enrolled in the prenatal part of the study. Most postnatal data were obtained by community health centers, which the participants visited as a part of the national routine health care program. Furthermore, parents were requested to fill out several questionnaires during the first years of their child's life regarding the health, growth, diet, behavior and development of the child. Finally, a number of studies were performed in collaboration with other birth cohorts. Most of these studies are part of the Early Growth Genes (EGG) and Early Genetics and Longitudinal Epidemiology (EAGLE) Consortium, of which the primary aim is to identify the new genetic variants of various growth and health outcomes in early life.

Outline of Thesis

In **chapter 2**, studies regarding the relationship between early fetal growth, birth outcomes and body composition during infancy and childhood are presented. The risk factors and outcomes associated with first-trimester fetal growth restriction are described (**chapter 2.1**), as well as the heritability of body size from fetal life to early childhood (**chapter 2.2**), the relationship between fetal growth and abdominal adiposity during infancy (**chapter 2.3 and chapter 2.4**), and the associations between fetal and postnatal growth rates (**chapter 2.5**).

Chapter 3 describes two studies (**chapter 3.1 and chapter 3.2**) in which we examine the associations of the obesity related gene *FTO* with early growth and body composition. Furthermore, we discuss two type 2 diabetes genes, *TCF7L2* (**chapter 3.3**) and *PPARγ-2* (**chapter 3.4**), and their association with early growth. **Chapter 4.1** describes a genome-wide association study on birth weight which yielded the discovery of two new genetic associations. In **chapter 4.2**, we further examine the associations of these two new candidates with fetal growth. Finally, **chapter 5** provides a review of the studies that have been performed regarding the fetal insulin hypothesis and the new insights that have been coming from the genome-wide association studies. Moreover, we discuss the usefulness of these new genetic findings for clinical practice.

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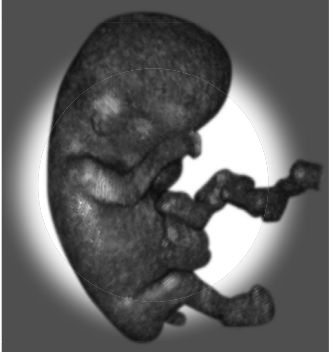
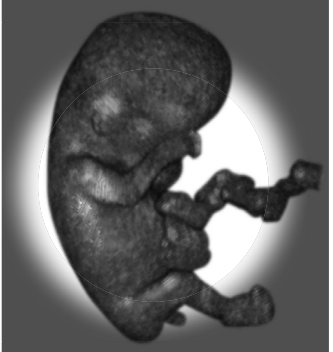


CHAPTER 2

Early growth and body composition



CHAPTER 2.1

Risk factors and outcomes associated with first-trimester fetal growth restriction

Dennis O Mook-Kanamori
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Adapted from JAMA;2010:303(6):527-534

Abstract

Background: Adverse environmental exposures lead to developmental adaptations in fetal life. The influences of maternal physical characteristics and lifestyle habits on first-trimester fetal adaptations and the postnatal consequences are not known. Our objective was to determine the risk factors and outcomes associated with first-trimester growth restriction.

Methods: We prospectively evaluated the associations of maternal physical characteristics and lifestyle habits with first-trimester fetal crown to rump length in 1,631 mothers with a known and reliable first day of last menstrual period and regular menstrual cycle. Subsequently, we assessed the associations of first-trimester fetal growth restriction with the risks of adverse birth outcomes and postnatal growth acceleration until the age of 2 years. The study was based in Rotterdam, the Netherlands. Mothers were enrolled between 2001 and 2005. First-trimester fetal growth was measured as fetal crown to rump length by ultrasound between a gestational age of 10 weeks 0 days and 13 weeks and 6 days. Main birth outcomes were preterm birth (gestational age < 37 weeks), low birth weight (< 2,500 grams) and small size for gestational age (lowest 5th birth centile). Postnatal growth was measured until the age of 2 years.

Results: In the multivariate analysis, maternal age was positively associated with first-trimester fetal crown to rump length (difference: 0.79 mm (95% CI: 0.41, 1.18) per standard deviation increase). Higher diastolic blood pressure and higher hematocrit levels were associated with a smaller crown to rump length (differences: -0.40 mm (95% CI: -0.74, -0.06) and -0.52 mm (95% CI: -0.90, -0.14) per standard deviation increase, respectively). Compared to non-smoking and optimal use of folic acid supplements, mothers who both smoked and did not use folic acid supplements had a smaller fetal crown rump length (difference: -3.84 mm (95% CI: -5.71, -1.98)). Compared to normal first-trimester fetal growth, first-trimester growth restriction was associated with increased risks of preterm birth (4.0% versus 7.2%), low birth weight (3.5% versus 7.5%) and small size for gestational age at birth (4.0% versus 10.6%) (Corresponding adjusted odds ratios: 2.12 (95% CI: 1.24, 3.61), 2.42 (95% CI: 1.41, 4.16) and 2.64 (95% CI: 1.64, 4.25)). Each standard deviation decrease in first trimester crown to rump length was associated with postnatal growth acceleration until the age of 2 years (increase: 0.139 SDS per 2 years (95% CI: 0.097 to 0.181).

Conclusions: Maternal physical characteristics and lifestyle habits were independently associated with early fetal growth. First-trimester fetal growth restriction was associated with an increased risk of adverse birth outcomes and growth acceleration in early childhood.

Introduction

Fetal developmental adaptations due to adverse environmental exposures may affect the structure, physiology and function of various organ systems leading to fetal growth restriction and increased risks of metabolic and cardiovascular disease in adulthood.[1,2] Human growth and development rates are highest during the first trimester of pregnancy, when essential fetal organ development is completed.[3] Adverse first-trimester fetal exposures might have permanent consequences for fetal and postnatal health.

First-trimester fetal crown to rump length is used in obstetric care practice for pregnancy dating assuming no growth variation.[4] Studies among women with a regular menstrual cycle suggested that maternal age, ethnicity and fetal sex are associated with fetal crown to rump length.[5,6] Maternal smoking, alcohol consumption and folic acid supplement use influence birth weight,[7,8] but their associations with first-trimester fetal growth are not known. Two studies suggested that first-trimester fetal growth restriction increases the risks of low birth weight and small size for gestational age.[9,10] The relationship between first-trimester fetal growth restriction and postnatal health outcomes remains unknown.

In a population-based prospective cohort study among mothers with a known first day of last menstrual period, we examined the associations of several maternal physical characteristics and lifestyle habits with first-trimester fetal growth. Subsequently, we examined whether first-trimester fetal growth restriction is associated with the risks of adverse birth outcomes and growth adaptations during fetal life and early childhood.

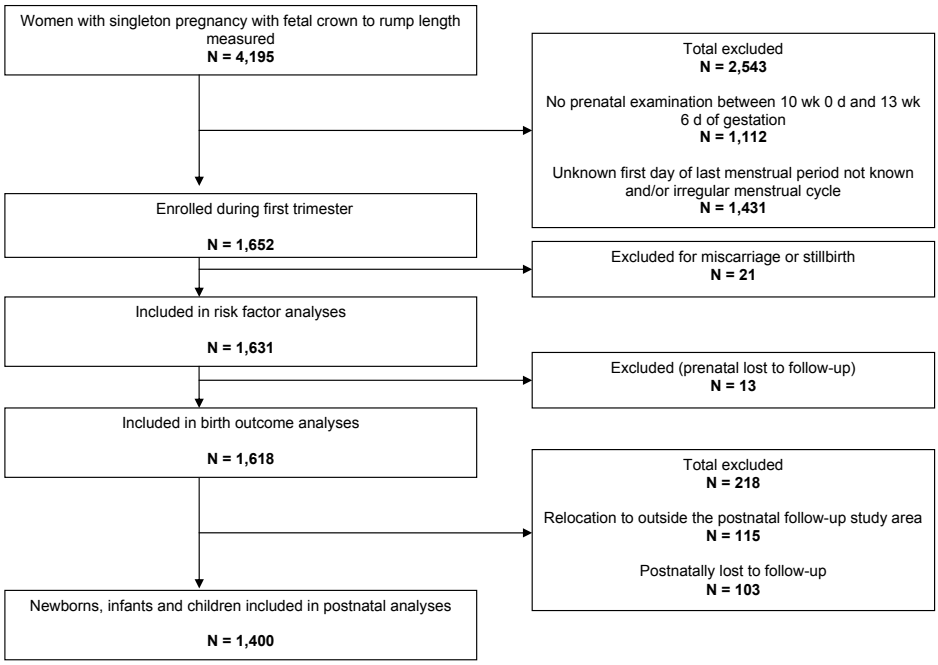
Methods

Study design and cohort

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, the Netherlands.[11] The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, the Netherlands. Written informed consent for both maternal and child data was obtained from all mothers.

Mothers enrolled between 2001 and 2005. Fetal crown to rump length was measured in 4,195 mothers with singleton pregnancies (Figure 1). Of these mothers, 3,083 had measurements within the recommended gestational age range of 10 weeks 0 days and 13 weeks 6 days.[12] Mothers who did not undergo prenatal examinations within this time frame (n=1,112) and those in whom pregnancy ended with miscarriage or still birth (n=21) were excluded, since gestational age at time of death with subsequent arrest of

Figure 1: Flow chart of participants in the study.



fetal growth could be ascertained.[13] Mother with neither a known and reliable first day of last menstrual period nor a regular menstrual cycle between 28 plus or minus 4 days were excluded (n=1,431), leaving 1,631 participants for the analyses. First day of the last menstrual period was obtained from the referring letter from the community midwife or hospital. This date was confirmed with the mother at the ultrasound visit and additional information on the regularity and cycle duration was obtained.

The prevalence of pre-eclampsia and (gestational) diabetes was 1.7% and 1.1%, respectively. Since exclusion of these women did not materially change our results, they were included in the analyses.

Maternal risk factors

Information on maternal age, educational level, smoking habits, alcohol use, folic acid supplement use, parity, and mode of conception were obtained through self-administered questionnaire at enrollment (response rate 93%). Since fetal growth is known to vary between ethnicities,[14] participating mothers were requested to give details regarding the country of birth of their parents. This information was used to classify participants' ethnic background according to Statistics Netherlands, as previously described in detail.[14] Weight and height were measured without shoes or heavy clothing and body mass index was calculated (weight/height^2 (kg/m^2)). Blood pressure was measured

with the validated Omron 907® automated digital oscillometric sphygmomanometer (OMRON Healthcare Europe B.V. Hoofddorp, the Netherlands). After being seated in an upright position for at least 5 minutes, the mean value of two blood pressure readings over a 60-second interval was documented. Hematocrit and hemoglobin levels were available from first-trimester blood samples (success rate 84%).

Fetal ultrasounds

In our research facility, we measured first-trimester (<14 weeks) crown to rump length in a true mid-sagittal plane with the genital tubercle and the fetal spine longitudinally in view.[15] The maximum length from cranium to the caudal rump was measured as a straight line. Transvaginal scanning was performed when there was limited visibility by transabdominal scanning. In second (median: 20.3 (90% range: 19.1-22.0) weeks) and third (median: 30.1 (90% range: 29.0-32.0) weeks) trimester, we measured fetal head circumference (HC), biparietal diameter (BPD), abdominal circumference (AC) and femur length (FL) to the nearest millimeter using standardized ultrasound procedures.[16] Estimated fetal weight (EFW) was calculated using the formula by Hadlock *et al*.[17] Ultrasound examinations were performed using an Aloka® model SSD-1700 (Tokyo, Japan) or the ATL-Philips® Model HDI 5000 (Seattle, WA, USA). Standard deviation scores (SDS) for all fetal growth characteristics were constructed.[16] Intra-class correlation coefficients for intraobserver and interobserver reproducibility of crown to rump length measurements were 0.998 and 0.995, respectively.[18]

Birth outcomes

Date of birth, birth anthropometrics (weight, length, head circumference) and offspring sex were obtained from community midwife and hospital registries. Gestational age and sex-adjusted standard deviation scores for birth weight, length and head circumference were constructed using growth standards from Usher and McLean.[19] Small size for gestational age at birth was defined within our study population (lowest 5th birth centile), preterm birth as a gestational age at birth less than 37 weeks and low birth weight as a birth weight less than 2,500 grams.

Postnatal growth

Well-trained staff in community health centers obtained postnatal growth characteristics (weight, length and head circumference) using standardized procedures.[11] Based on the routine health care program, periodic visits scheduled for eight age periods (1, 2, 3, 4, 6, 11, 14, and 24 months). Actual median (90% range) ages (by weeks) were: 4.7 (3.6, 6.6);

9.7 (8.7, 12.6); 14.4 (13.0, 16.9); 19.0 (17.4, 21.2); 26.7 (23.7, 31.0); 47.6 (43.5, 53.0); 61.8 (59.3, 68.2); and 107.1 (102.1, 118.2). For postnatal growth characteristics, we obtained standard deviation scores using Dutch reference growth curves (Growth Analyser 3.0, Dutch Growth Research Foundation, Rotterdam, the Netherlands).

Statistical analysis

First, we performed a nonresponse analysis by comparing characteristics of mothers who were included in the analyses to those who were excluded due to an unknown or unreliable first day of last menstrual period or an irregular menstrual cycle using Student's T-tests, Mann-Whitney's U-tests and Chi-square tests. Also, we compared birth characteristics of children included in the postnatal analyses to those who were not.

Second, we created gestational age-adjusted SDS for each individual fetal measurement in our study (Supplementary Appendix and Figure S2). We used fetal sex and gestational age adjusted linear regression models to assess the associations of each determinant with first-trimester fetal crown to rump length separately. To enable comparison of the effect estimates between risk factors, we present our results as change per SDS for continuous variables. Subsequently, all factors associated with crown to rump length were included in one multivariate linear regression model. We also examined interactions between lifestyle exposures (smoking, alcohol use and folic acid supplement use). Since the timing of ovulation is dependent of the duration of the menstrual cycle, these models were adjusted for the duration of the last menstrual cycle.

Third, we examined the associations of first-trimester fetal growth restriction, defined as gestational age adjusted crown to rump length in the lowest 20% of the population, with the risk of adverse birth outcomes (preterm birth, small size for gestational age, and low birth weight) using univariate logistic regression models. To account for potential confounding, these models were subsequently adjusted for factors associated with fetal crown to rump length.

Finally, we studied the associations of first-trimester fetal crown to rump length with growth characteristics in second and third trimester and from birth until the age of 24 months using sex adjusted linear regression models. To assess the associations of first-trimester fetal crown to rump length with longitudinally measured growth rates in childhood, we performed unbalanced repeated measures regression analysis (Appendix).

With a sample size of 1,631 subjects and assuming a power of 0.80 and a significance level of 0.05 (2-sided), we were able to detect an effect of 0.07 standard deviations. When multiple comparisons were performed, the significance level was adjusted using Bonferroni's correction. For all analyses, missing values were imputed with the mean for continuous variables or additional category for categorical variables. We found similar results based on imputed and complete datasets. Statistical analyses were performed

using the Statistical Analysis System version 9.1.3 (SAS, Stata corporation, College station, TX, USA) and the Statistical Package of Social Sciences version 15.0 (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics

Maternal and fetal characteristics are shown in Table 1. Nonresponse analysis showed that mothers with a known first day of the last menstrual period and a reliable menstrual

Table 1: Maternal and fetal characteristics (n=1,631)

Maternal characteristics	
Age at intake (years)	31.4 (21.9 – 37.9)
Gestational age at intake (weeks)	12.4 (0.8)
Height (cm)	169 (7.0)
Weight (kg)	67.0 (53.0 – 92.5)
Body mass index (kg/m^2)	23.5 (19.4 – 32.0)
Blood pressure at intake	
Mean systolic (mmHg)	117 (12.4)
Mean diastolic (mmHg)	69 (9.5)
Mean hematocrit level (%)	36.7 (2.5)
Mean hemoglobin level (gram/dL)	12.4 (0.9)
Educational level	
Primary (%)	93 (6.0)
Secondary (%)	617 (39.6)
Higher (%)	847 (54.4)
Ethnicity	
Dutch/Caucasian (%)	1127 (71.6)
Surinamese (%)	88 (5.6)
Turkish (%)	97 (6.2)
Moroccan (%)	56 (3.6)
Indonesian (%)	48 (3.0)
Others (%)	158 (10.0)
Smoking (% yes)	355 (24.3)
Alcohol (% yes)	888 (60.5)
Folic acid supplement use (% yes)	1117 (85.1)
Parity (% primiparous)	962 (59.3)
Conception (% spontaneous)	1552 (98.9)

Values represent means (SD), median (90% range) or number of subjects (%).

Table 1 (continued): Maternal and fetal characteristics (n=1,631)

Fetal and characteristics	
First-trimester crown to rump length (mm)	60.9 (11.4)
Sex (% boys)	817 (50.5)
Birth head circumference (cm)	34.0 (1.7)
Birth length (cm)	50.4 (2.4)
Birth weight (grams)	3454 (565)
Gestational age at birth (weeks)	40.1 (37.1 – 42.0)
Preterm birth (< 37 weeks) (%)	75 (4.6)
Small for gestational age (< 5th birthcentile) (%)	86 (5.3)
Low birth weight (< 2,500 grams) (%)	69 (4.3)

Values represent means (SD), median (90% range) or number of subjects (%).

cycle were older, more highly educated, more often white and more frequently users of folic acid supplements (all p<0.001) (Table S1). Of all newborns, infants, and children, 7% were excluded from postnatal follow-up studies because of relocation outside of the study area, and 6% were lost to follow-up for other reasons. Postnatal follow-up data were available in the remaining 87% of all newborn, infants, and children in the study. Those who were not included in the postnatal analyses had a lower birth weight (difference: 156 grams (95% CI: 61, 251);p<0.001) and a smaller size for gestational age at birth (difference 0.50 weeks (95% CI: 0.18, 0.83);p=0.009) compared with those included in these analyses (Table S2).

Risk factors of first-trimester fetal growth variation

In the univariate analyses, higher maternal age, folic acid supplement use and having had a previous childbirth were positively associated with fetal crown to rump length (Table 2). Higher diastolic blood pressure and hematocrit level, a secondary education only, Surinamese ethnicity and smoking were associated with a shorter fetal crown to rump length. Maternal anthropometrics were not associated with fetal crown to rump length.

In the multivariate analyses, the associations of maternal age (0.10 SDS (95% CI: 0.05, 0.16), p<0.001) per SD increase in age), diastolic blood pressure (-0.05 SDS (95% CI: -0.10,

Table 2: Maternal risk factors of first-trimester variation in fetal crown to rump length adjusted for sex and gestational age (n=1,631).

Risk factor	**Effect size (mm)**	**P-value**	**Effect size (SDS)**	**P-value**
Age at intake (1 SD= 4.68 years)	0.840 (0.506, 1.175)	< 0.001	0.111 (0.064, 0.159)	< 0.001
Height (1 SD= 7.07 cm)	0.165 (-0.169, 0.499)	0.33	0.016 (-0.032, 0.064)	0.50
Weight (1 SD= 12.42 kg)	0.085 (-0.252, 0.423)	0.62	0.005 (-0.044, 0.053)	0.85
Body mass index (1 SD= 4.08 kg/m^2)	-0.009 (-0.353, 0.332)	0.96	-0.006 (-0.055, 0.043)	0.81

Table 2 (continued): Maternal risk factors of first-trimester variation in fetal crown to rump length adjusted for sex and gestational age (n=1,631).

Risk factor	Effect size (mm)	P-value	Effect size (SDS)	P-value
Blood pressure at intake				
Mean systolic (1 SD= 12.38 mm Hg)	-0.306 (-0.635, 0.023)	0.07	-0.045 (-0.092, 0.002)	0.06
Mean diastolic (1 SD= 9.46 mm Hg)	-0.436 (-0.770, -0.103)	0.01	-0.061 (-0.109, -0.013)	0.01
Hematocrit level (1 SD= 2.50 %)	-0.573 (-0.945, -0.201)	0.003	-0.073 (-0.126, -0.019)	0.008
Hemoglobin level (1 SD= 0.94 grams/dL)	-0.557 (-0.911, -0.203)	0.002	-0.064 (-0.115, -0.014)	0.01
Educational level				
Primary	0.747 (-0.706, 2.201)	0.31	0.105 (-0.104, 0.313)	0.33
Secondary	-0.762 (-1.465929, -0.058)	0.03	-0.095 (-0.255, 0.006)	0.07
Higher	-0- (reference)		-0- (reference)	
Ethnicity				
Dutch/Caucasian	-0- (reference)		-0- (reference)	
Surinamese	-2.216 (-3.688, -0.744)	0.003	-0.259 (-0.469, -0.048)	0.02
Turkish	-0.292 (-1.691, 1.108)	0.68	-0.046 (-0.248, 0.155)	0.65
Moroccan	1.229 (-0.581, 3.039)	0.18	0.212 (-0.076, 0.485)	0.11
Indonesian	1.302 (-0.646, 3.250)	0.19	0.204 (-0.076, 0.485)	0.15
Others	0.439 (-0.694, 1.572)	0.45	0.078 (-0.084, 0.239)	0.35
Smoking				
No	-0- (reference)		-0- (reference)	
Yes (all)	-1.195 (-2.006, -0.385)	0.004	-0.161 (-0.277, -0.045)	0.007
Yes (< 5 per day)	-0.905 (-1.965, 0.155)	0.09	-0.129 (-0.280, 0.022)	0.09
Yes (5-9 cigarettes per day)	-0.825 (-2.261, 0.621)	0.26	-0.117 (-0.323, 0.089)	0.27
Yes (>10 cigarettes per day)	-1.738 (-3.376, -0.100)	0.04	-0.208 (-0.443, 0.027)	0.08
Alcohol				
Yes	0.402 (-0.309, 1.114)	0.27	0.050 (-0.052, 0.152)	0.34
No	-0- (reference)		-0- (reference)	
Folic acid supplement use				
Yes	-0- (reference)		-0- (reference)	
No	-1.102 (-2.138, -0.066)	0.04	-0.140 (-0.288, 0.008)	0.06
Parity				
0	-0- (reference)		-0- (reference)	
1+	0.834 (0.161, 1.506)	0.02	0.113 (0.017, 0.210)	0.02
Spontaneous conception				
Yes	-0- (reference)		-0- (reference)	
No	-0.607 (-3.743, 2.529)	0.70	-0.138 (-0.588, 0.312)	0.55

Values represent regression coefficients (95% confidence interval) and their corresponding p-values. For continuous variables the effect estimates represent the change in fetal crown to rump length (mm or standard deviation score (SDS)) per increase of standard deviation of the risk factor. For categorical or dichotomous variables, the effect estimates represent the difference in fetal crown to rump length compared to the reference group. Variables included in the model: fetal sex and gestational age.

-0.01), p=0.03) per SD increase in blood pressure, hematocrit level (-0.07 SDS (95% CI: -0.12, -0.01, p=0.02) per SD increase in hematocrit level, smoking (-0.13 SDS (95% CI: -0.25, -0.01), p=0.03) and folic acid supplement use (0.17 SDS (95% CI: 0.33, 0.01), p=0.03) with fetal crown to rump length remained significant (Table 3). After multiple testing adjustment (six independent risk factors), the associations with diastolic blood pressure, smoking and folic acid supplement use were no longer significant. We also observed a dose-response association between the number of cigarettes smoked daily by mother and fetal crown to rump length (p=0.04). A stratified analysis according to gestational age indicated no interaction between gestational age and risk factors except for maternal age, in which the effect size increased with increasing gestational age (p=0.002) (Table S3).

We observed an interaction of maternal smoking and folic acid intake (p<0.001), indicating that mothers who smoked and did not use folic acid supplements had a smaller fetal crown to rump length growth of -0.52 SDS (95% CI: -0.78, -0.25) or -3.84 mm (95% CI: -5.71, -1.98) compared to non-smoking mothers who used folic acid supplements (Figure S2). We did not observe other interactions between smoking, alcohol use and folic acid supplement use (all p >0.1).

Table 3: Maternal risk factors of first-trimester variation in fetal crown to rump length using multivariate analysis (n=1,631).

Risk factor	Effect size (mm)	P-value	Effect size (SDS)	P-value
Age at intake (1 SD= 4.68 years)	0.793 (0.409, 1.178)	< 0.001	0.103 (0.048, 0.158)	< 0.001
Diastolic blood pressure (1 SD= 9.52 mm Hg)	-0.398 (-0.737, -0.059)	0.02	-0.054 (-0.103, -0.006)	0.03
Hematocrit level (1 SD= 2.50 %) §	-0.523 (-0.904, -0.142)	0.006	-0.065 (-0.120, -0.011)	0.02
Hemoglobin level (1 SD= 0.946 grams/dL) §	-0.521 (-0.888, -0.155)	0.005	-0.061 (-0.113, -0.008)	0.02
Smoking (No = reference)				
Yes (all)	-0.975 (-1.792, -0.158)	0.02	-0.127 (-0.245, -0.010)	0.03
Yes (< 5 per day)	-0.624 (-1.671, 0.423)	0.24	-0.081 (-0.232, 0.069)	0.29
Yes (5-9 cigarettes per day)	-0.662 (-2.086, 0.762)	0.36	-0.092 (-0.297, 0.112)	0.38
Yes (10+ cigarettes per day)	-1.253 (-2.871, 0.364)	0.13	-0.144 (-0.377, 0.089)	0.23
Folic acid supplement use (Yes = reference)				
No	-1.327 (-2.409, -0.244)	0.02	-0.170 (-0.325, -0.014)	0.03
Parity (-0- = reference)				
1+	0.272 (-0.430, 0.974)	0.45	0.038 (-0.063, 0.139)	0.57

§ Hematocrit and hemoglobin levels were added independently into the model due to the high correlation between these two risk factors.

Values represent regression coefficients (95% confidence interval) and their corresponding p-values. For continuous variables the effect estimates represent the change in fetal crown to rump length (mm or standard deviation score (SDS)) per increase of standard deviation of the risk factor. For categorical or dichotomous variables, the effect estimates represent the difference in fetal crown to rump length compared to the reference group. Variables included in the model: duration of last menstrual cycle, gestational age, fetal sex, age of mother at intake, blood pressure, hematocrit level, educational level, ethnicity, smoking, folic acid use and parity.

First-trimester fetal growth restriction and adverse birth outcomes

Small first-trimester fetal crown to rump length, defined as the lowest 20%, was associated with an increased risk of being born preterm (4.0% vs. 7.2%), small size for gestational age (4.0% vs. 10.6%) or with a low birth weight (3.5% vs. 7.5%) (Table 4). Using the same data, sensitivity analyses with a cut-off of the lowest 10% instead of the lowest 20% for crown to rump length showed similar effect estimates (preterm birth 4.2% vs. 8.8%; OR 2.43 (95% CI: 1.28, 4.59, p=0.006), for small size for gestational age at birth 4.5% vs. 12.5%; OR 2.50 (95% CI: 1.42, 4.41, p=0.001) and for low birth weight 3.7% vs. 9.4%; OR 2.70 (95% CI: 1.43, 5.09, p=0.002)).

Table 4: Associations of first-trimester fetal growth restriction with adverse birth outcomes (n=1,631).

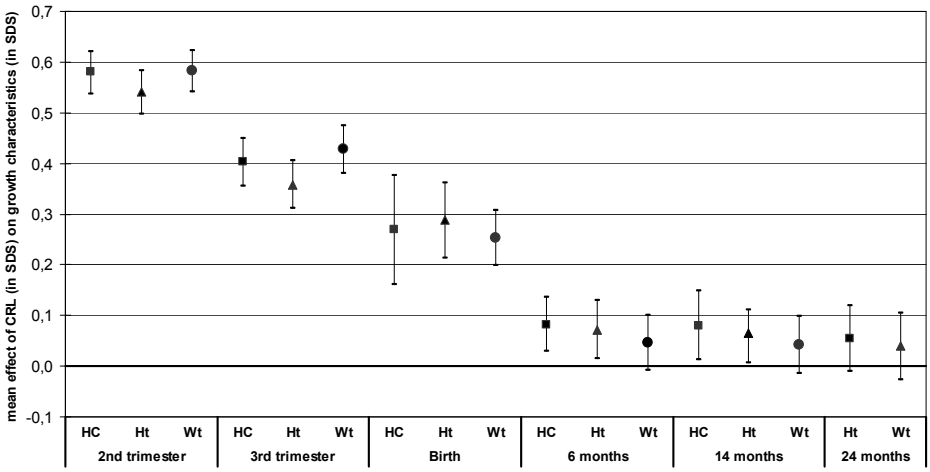
Crown to rump Length (SDS)	Preterm birth		Unadjusted Odds Ratio	Adjusted Odds Ratio
	< 37 weeks	>= 37 weeks		
< 20th percentile (%)	23 (7.2)	298 (92.8)	1.85 (1.11, 3.07)	2.12 (1.24, 3.61)
> 20th percentile (%)	52 (4.0)	1245 (96.0)	p = 0.02	p = 0.006
Crown to rump Length (SDS)	**Small size for gestational age**		**Unadjusted Odds Ratio**	**Adjusted Odds Ratios**
	< 5th birthcentile	**>= 5th birthcentile**		
< 20th percentile (%)	34 (10.6)	287 (89.4)	2.84 (1.81, 4.45)	2.64 (1.64, 4.25)
> 20th percentile (%)	52 (4.0)	1245 (96.0)	p < 0.001	p < 0.001
Crown to rump Length (SDS)	**Low birth weight**		**Unadjusted Odds Ratio**	**Adjusted Odds Ratio**
	< 2,500 grams	**>= 2,500 grams**		
< 20th percentile (%)	24 (7.5)	297 (92.5)	2.24 (1.35, 3.75)	2.42 (1.41, 4.16)
> 20th percentile (%)	45 (3.5)	1252 (96.5)	p = 0.002	p = 0.001

Odds ratios were based on multiple logistic regression models. Variables included in the model: duration of last menstrual cycle, fetal sex, age of mother at intake, educational level, ethnicity, parity, diastolic blood pressure, hematocrit level, smoking and folic acid supplement use.

First-trimester fetal growth restriction and growth in fetal life and early childhood

First-trimester fetal crown to rump length was positively associated with second and third trimester head circumference, femur length and estimated fetal weight (Figure 2). The associations of first-trimester fetal crown to rump length with biparietal diameter and abdominal circumference are shown in Table S4. First-trimester crown to rump length was associated with postnatal weight until the age of 11 months (0.061 (95% CI: 0.005, 0.117), p=0.03) and with postnatal head circumference and length until the age of 14 months (0.065 (95% CI: 0.007, 0.122), p=0.03) and 0.080 (95% CI: 0.012, 0.148), p=0.02, respectively). No associations between crown to rump length and growth parameters at the age of 2 years were found. Each SD decrease in first-trimester fetal crown to rump length was associated with an accelerated growth rate in weight and height during the

Figure 2: Associations of first-trimester fetal crown to rump length with growth characteristics in later pregnancy and early childhood.



Values are regression coefficients (95% confidence interval) and represent the change (standard deviation score (SDS)) of head circumference (HC), (femur) length (Ht) and (estimated fetal) weight (Wt) per increase of 1 standard deviation in first-trimester fetal crown to rump length. All models adjusted for fetal sex. Head circumference was not available at the age of 24 months.

first two years (increase of 0.139 SDS / 2 years (95% CI: 0.097, 0.181, p<0.001) and 0.128 SDS / 2 years (95% CI: 0.085, 0.173, p<0.001), respectively). These associations were independent of birth weight.

Discussion

This study showed that maternal physical characteristics and lifestyle habits are associated with first-trimester crown to rump length, as a measure of first-trimester fetal growth. Shorter first-trimester crown to rump length was associated with increased risks of preterm birth, small size for gestational age at birth and low birth weight. Furthermore, shorter first-trimester crown to rump length was associated with accelerated growth rates in early childhood.

We used the first day of the last menstruation period to determine gestational age at crown to rump length measurement. To avoid misclassification, we included only pregnant women with a known and reliable first day of the last menstruation period and a regular menstrual cycle of approximately 28 days. Misclassification of gestational age might still be an issue since the postconceptional age at the time of ultrasound is dependent of the timing of ovulation and implantation, which we were unable to measure. For example, the duration of the follicular phase, after which ovulation occurs, has

been shown to also be associated with several maternal factors, such as maternal age and smoking.[20] Furthermore, the dating of the last menstrual period can be confounded by recall bias.[21] However, all our results remained after adjustment for the duration of last menstrual cycle, which is highly associated with the timing of ovulation. Moreover, even with a known and reliable last menstrual period, a certain fraction of women with regular cycles have early or delayed ovulation. Nonetheless, restriction to participants who had a gestational age based on last menstruation within 7 days of a gestational age based on crown to rump length (93%) did not materially change our effect estimates (data not shown).

Mothers with a known and reliable last menstrual period were on average older, taller, more likely to be higher educated and Dutch, consume alcohol during pregnancy and use folic acid supplements than those with an unreliable menstrual cycle. Our effect estimates would be biased if the associations between risk factors and first-trimester fetal growth would differ between individuals included and not included in the analysis. This seems unlikely.[22] Children lost to follow-up were on average smaller at birth, which is a risk factor for postnatal growth acceleration.[23] This might have led to an underestimation of the postnatal growth rates in children with first-trimester fetal growth restriction. Restricting the analyses on birth outcomes to those children with complete fetal and postnatal data did not materially change the associations with the risks of adverse birth outcomes (data not shown).

Birth weight is the end-point of different fetal growth patterns and adverse exposures that may influence fetal growth as early as the first-trimester. Previous studies suggested that maternal age, black race and male sex are associated with longer first-trimester crown to rump length[5,6] We found negative associations of higher diastolic blood pressure and hematocrit levels with first-trimester crown to rump length. Higher blood pressure in early pregnancy is known to be associated with an increased risk of developing placenta related problems such as pre-eclampsia and fetal growth restriction in later pregnancy.[24] A possible explanation is that higher hematocrit levels are indicative of a lower circulating plasma volume, which subsequently might lead to a suboptimal placental perfusion.[25] Finally, maternal anthropometrics were not associated with first-trimester crown to rump length, indicating that the previously found associations between maternal weight and birth weight develop in later pregnancy.[26]

Smoking and the non-use of folic acid supplements were associated with shorter first-trimester growth. Folic acid serves as a substrate for various cellular processes, such as cell division and apoptosis, which are implicated in fetal and placental growth and development.[27] Furthermore, both smoking and folic acid seem to be involved in DNA methylation, which may subsequently affect early fetal and postnatal growth.[28] There was a strong additive interaction between smoking and folic acid supplement intake. Jauniaux *et al*.[29] described that folic acid levels in serum levels and coelomic fluid were

lower in smokers than non-smokers, suggesting that smoking may lead to an impaired bioavailability of folic acid. Smoking during pregnancy also induces morphological and functional changes in the placenta, leading to a reduction of fetal-placental blood flow.[30] Further studies on the effects of these lifestyle factors on early markers of abnormal placentation, such as pregnancy-associated plasma protein A and free human chorionic gonadotropin-β, will allow us to examine in greater detail what role placental function plays in the relationship between these factors and early fetal growth.[31]

First-trimester fetal growth restriction was associated with increased risks of prematurity, small size for gestational age at birth and low birth weight, which are associated with increased perinatal mortality and morbidity. These findings are consistent with results from two previous studies, one in spontaneously conceived pregnancies and the other in pregnancies resulting from assisted reproductive technology.[9,10] Smaller first-trimester crown to rump length may be an intermediate in the pathway between adverse maternal risk factors, such as smoking and non-use of folic acid supplements, and growth variation in later pregnancy and possibly later life.[32]

Our results indicate that first-trimester growth variation is associated with growth parameters in early childhood. Smaller first-trimester fetal crown to rump length led to compensatory accelerated postnatal growth. Subsequently, at the age of 24 months there was no longer an association between fetal growth restriction and postnatal growth parameters. Increased postnatal growth rate is a well-established risk factor for metabolic and cardiovascular disease in later life.[23,33] It could be that growth as early as in the first-trimester of pregnancy is associated with disease in adulthood, although longer follow-up studies are necessary to examine this relationship.

In conclusion, our study demonstrates that first-trimester fetal growth is associated with several maternal physical characteristics and lifestyle habits. First-trimester growth restriction is associated with higher risks of adverse birth outcomes and accelerated postnatal growth rates. Further studies are needed to assess the associations of first-trimester growth variation on the risks of disease in later childhood and adulthood.

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Appendix

Crown rump length standard deviation scores (SDS)

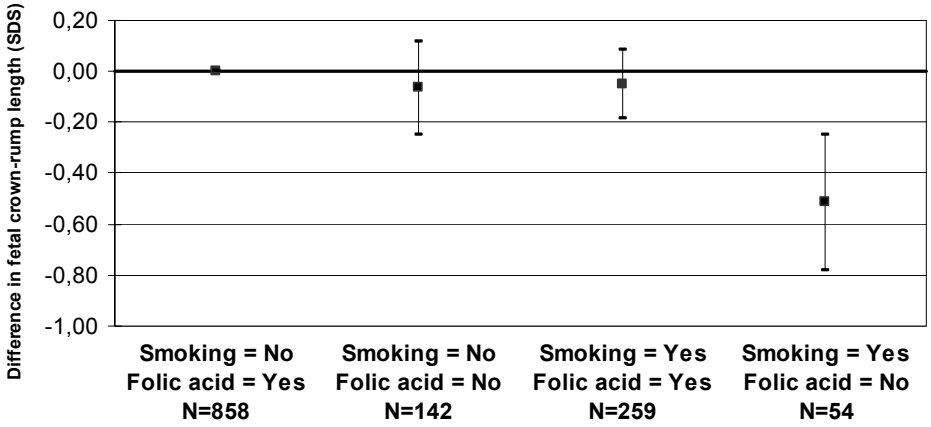
The statistical analyses for fetal growth measurements (crown to rump length, head circumference, biparietal diameter, abdominal circumference, femur length and estimated weight) are partly based on standard deviation scores (SDS). These scores enable adjustment for gestational age (GA) avoiding the inclusion of non-linear functions of GA in models. Our approach for developing reference models for constructing SDS was based on the LMS model of Cole and Green,[1] as implemented in the GAMLSS software of Rigby and Stasinopoulos.[2] The LMS model of Cole and Green assumes that, after a Box-Cox transformation, crown to rump length (or any other fetal growth measurement) has a normal distribution. The acronym LMS stands for lambda, mu, sigma. Mean and standard deviation of the distribution are allowed to change with GA, possibly by smooth curves, described by polynomials of splines. The formula for creating SD scores is:

$$z = \frac{(y/\mu(x))^{\lambda(x)} - 1}{\lambda(x)\sigma(x)},$$

where x stands for GA and y for crown to rump length (CRL). The model comprises three curves: $\lambda(x)$ for the parameter of the Box-Cox transformation, $\mu(x)$ for the mean of the transformed crown to rump length and $\sigma(x)$ for its standard deviation. Once the curves $\lambda(x)$, $\mu(x)$ and $\sigma(x)$ have been obtained, one can fill in any data pair (x, y) and obtain the standard deviation score z. The curves in the LMS can be flexible, but they might also be straight lines or even constants depending on the data. The GAMLSS software of Rigby and Stasinopoulos allows one to experiment with a large number of settings and to judge the quality of models by means of the log-likelihood and Akaike's information criterion (AIC).

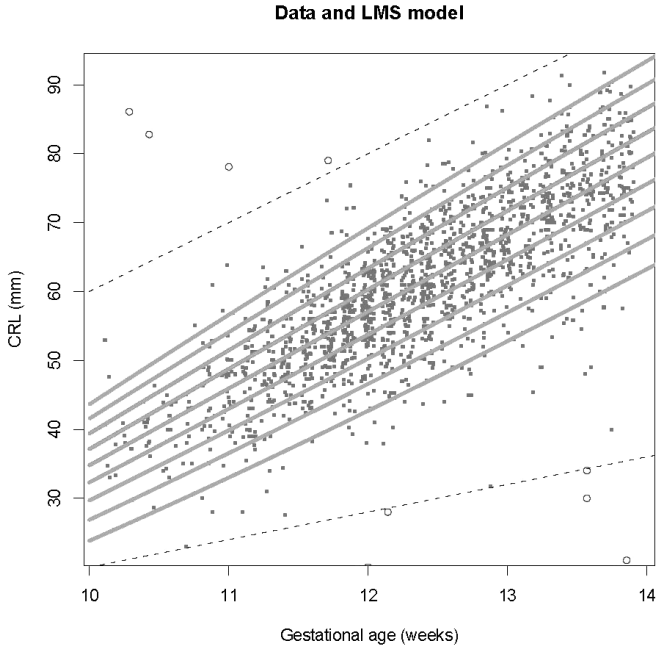
Figure S2 shows the data and curves of constant SDS for crown to rump length. All crown to rump length measurements are in the range between 10 weeks 0 days and 13 weeks 6 days for GA. The dataset contained some evident outliers. To remove them, two linear boundaries were defined, one connecting GA = 10 weeks, CRL = 20 mm with GA = 13+6 weeks, CRL = 35 mm, the other, connecting GA = 10 weeks, CRL = 60 mm with GA = 13+6 weeks, CRL = 95 mm. Data points outside these boundaries were removed from the dataset, as indicated by circles in the plot. The SDS curves in Figure S2 are based on an LMS model in which λ is constant, μ is linear in GA, as well as the logarithm of σ. The estimated value of λ is 1.893, indicating skewness to the left. This number is close to 2, suggesting that the squares of crown to rump length has a normal distribution with mean and SD that change with GA.

Figure S1: Association of maternal smoking and folic acid supplement use with first-trimester fetal crown to rump length.



Values are regression coefficients (95% confidence interval) and reflect the association of maternal smoking and folic acid supplement use with first-trimester fetal crown to rump length (measured as gestational age adjusted standard deviation score (SDS)).
Variables included in the model: duration of last menstrual cycle, fetal sex, age of mother, diastolic blood pressure, hematocrit level, educational level, ethnicity and parity.
Interaction between maternal smoking and folic acid supplement use ($p<0.001$).

Figure S2: Fetal crown to rump length according to gestational age with constant level standard deviation score curves.



Data and constant level SDS curves, based on the LMS model (SDS levels -2, -1.5, -1, -0.5, 0, 0.5, 1, 1.5, 2). Outliers indicated by circles. The broken thin straight lines represent the outlier boundaries.

Repeated measures regression analysis

These analyses were performed using the PROC MIXED module for unbalanced repeated measurements from the Statistical Analysis System version 9.1.3 (SAS, Stata corporation, College station, TX, USA). This regression technique takes the correlation of multiple measurements within one subject into account and assesses both the time-independent and time-dependent effect of crown to rump length on postnatal growth.[3] These models were adjusted for birth weight (SDS), since we wanted to assess the effect of first-trimester crown to rump length on postnatal growth rate independent of size at birth and can best be written as:

Weight (SDS) = β_0 + β_1*age (months) + β_2*crown to rump length (SDS) + β_3*crown to rump length*age + β_4*birth weight (SDS) + β_5*sex.

Height (SDS) = β_0 + β_1*age (months) + β_2*crown to rump length (SDS) + β_3*crown to rump length*age + β_4*birth weight (SDS) + β_5*sex.

In these models, the term including 'β_0' reflects the intercept. The terms including 'β_1 and β_2' represent the independent effects of age (months) and crown to rump length (SDS) on postnatal weight or height (SDS), respectively. The term including 'β_3' reflects the change in postnatal growth rate for weight or height (SDS/month) for each increase of 1 SD in crown to rump length. All models were adjusted for birth weight (SDS) and sex ('β_4' and 'β_5').

Table S1: Characteristics of mothers included and excluded* from the study.

Maternal characteristics	Included n=1,631	Excluded n=1,431	P-value
Age at intake (years)	31.4 (21.9 – 37.9)	30.0 (20.7 – 37.0)	< 0.001
Gestational age at intake (weeks)	12.4 (0.8)	N/A	N/A
Height (cm)	169 (7.0)	167 (7.4)	< 0.001
Weight (kg)	67.0 (53.0 – 92.5)	66.0 (51.0 – 95.0)	0.08
Body mass index (kg/m^2)	23.5 (19.4 – 32.0)	23.4 (19.1 – 34.1)	0.86
Blood pressure at intake			
Mean systolic (mmHg)	117 (12.4)	116 (12.3)	0.20
Mean diastolic (mmHg)	69 (9.4)	69 (9.7)	0.62
Mean hematocrit level (%)	36.7 (2.5)	36.6 (2.4)	0.41
Mean hemoglobin level (gram/dL)	12.4 (0.9)	12.4 (0.9)	0.21
Educational level			
Primary (%)	93 (6.0)	119 (9.1)	< 0.001
Secondary (%)	617 (39.6)	610 (46.6)	
Higher (%)	847 (54.4)	579 (44.3)	

Table S1 (continued): Characteristics of mothers included and excluded* from the study.

Maternal characteristics	Included n=1,631	Excluded n=1,431	P-value
Ethnicity			
Dutch/Caucasian (%)	1127 (71.6)	844 (63.4)	< 0.001
Surinamese (%)	88 (5.6)	132 (9.9)	
Turkish (%)	97 (6.2)	118 (8.9)	
Moroccan (%)	56 (3.6)	61 (4.6)	
Indonesian (%)	48 (3.0)	40 (3.0)	
Others (%)	158 (10.0)	136 (10.2)	
Smoking (% yes)	355 (24.3)	321 (26.3)	0.23
Alcohol (% yes)	888 (60.5)	659 (53.8)	< 0.001
Folic acid supplement use (% yes)	1117 (85.1)	840 (79.8)	< 0.001
Parity (% primiparous)	962 (59.3)	825 (58.6)	0.31
Conception (% spontaneous)	1552 (98.9)	1310 (98.6)	0.62

Values represent means (SD), median (90% range) or number of subjects (%)
** Mothers included in the analyses had a known and reliable date of first day of last menstrual period, regular menstrual cycle of 28 +/- 4 days, visit between 10+0 and 13+6 weeks of gestation and no miscarriage or perinatal death. Mothers excluded from the analyses had an unknown or unreliable date first day of last menstrual period or an irregular menstrual cycle.*
Differences were tested using Student's T-test or Mann-Whitney U-test for continuous variables and Chi-square test for categorical variables.

Table S2: Birth characteristics of children included and excluded* from the postnatal follow up analyses.

Fetal and characteristics	Included n=1,400	Excluded n=218	P-value
Sex (% boys)	709 (50.6)	107 (50.2)	0.94
Birth weight (grams)	3474 (545)	3318 (671)	< 0.001
Birth length (cm)	50.4 (2.4)	49.6 (2.8)	0.001
Birth head circumference (cm)	34.0 (1.7)	33.9 (1.5)	0.84
Gestational age at birth (weeks)	40.1 (37.1 – 42.1)	40.0 (35.6 – 42.0)	0.009

Values represent means (SD), median (90% range) or number of subjects (%).
** Children excluded from the postnatal follow up analyses analyses were either living outside the postnatal follow-up study area (7%, n=115) or were lost to follow-up postnatally (6%, n=103).*
Differences were tested using Student's T-test or Mann-Whitney U-test for continuous variables and Chi-square test for categorical variables.

Table S3: Risk factors of first-trimester variation in fetal crown to rump length using multivariate analysis stratified by gestational age (n=1,631).

Risk factor	Effect size (SDS) 10+0 to 11+6 weeks N = 483	Effect size (SDS) 12+0 to 12+6 weeks N = 711	Effect size (SDS) 13+0 to 13+6 weeks N = 437	P-value interaction Age*Risk Factor
Age at intake (1 SD= 4.68 years)	0.089 (-0.027, 0.204)	0.103 (0.020, 0.185)	0.079 (-0.019, 0.177)	0.002
Diastolic blood pressure (1 SD= 9.52 mm Hg)	0.010 (-0.097, 0.117)	-0.085 (-0.155, -0.015)	-0.069 (-0.156, 0.018)	0.41
Hematocrit level (1 SD= 2.50 %) §	-0.036 (-0.155, 0.084)	-0.054 (-0.133, 0.024)	-0.104 (-0.204, -0.005)	0.84
Hemoglobin level (1 SD= 0.946 grams/dL) §	-0.003 (-0.107, 0.101)	-0.066 (-0.146, 0.015)	-0.110 (-0.206, -0.015)	0.55
Smoking (No = reference)				
Yes (all)	0.002 (-0.233, 0.237)	-0.165 (-0.341, 0.012)	-0.232 (-0.447, -0.017)	0.44
Folic acid supplement use (Yes = reference)				
No	-0.008 (-0.354, 0.338)	-0.290 (-0.514, -0.065)	-0.112 (-0.394, 0.169)	0.63
Parity (-0- = reference)				
1+	-0.007 (-0.212, 0.198)	0.065 (-0.083, 0.213)	0.039 (-0.149, 0.228)	0.38

§ Hematocrit and hemoglobin level were added independently into the model due to the high correlation between these two risk factors.

Variables represent regression coefficients (95% confidence interval). For continuous variables the effect estimates represents the change in fetal crown to rump length (mm or standard deviation score (SDS)) per increase of standard deviation of the risk factor. For categorical or dichotomous variables, the effect estimates represents the difference in fetal crown to rump length compared to the reference group. Variables included in the model: duration of last menstrual cycle, fetal sex, age of mother at intake, diastolic blood pressure, hematocrit level, educational level, ethnicity, smoking, folic acid supplement use and parity. All effect estimates are also adjusted for gestational age at measurement.

Table S4: Associations of first-trimester fetal crown to rump length with fetal growth characteristics in later pregnancy and early childhood.

Age	Head circumference	p-value	Biparietal diameter	p-value	(Femur) length / height	p-value	(Estimated fetal) weight	p-value	Abdominal circumference	p-value
2nd trimester	0.580 (0.538, 0.621)	p<0.001	0.503 (0.458, 0.547)	p<0.001	0.541 (0.498, 0.583)	p<0.001	0.582 (0.542, 0.623)	p<0.001	0.497 (0.452, 0.542)	p<0.001
3rd trimester	0.403 (0.356, 0.450)	p<0.001	0.343 (0.295, 0.391)	p<0.001	0.358 (0.311, 0.406)	p<0.001	0.428 (0.381, 0.474)	p<0.001	0.370 (0.322, 0.418)	p<0.001
Birth	0.269 (0.161, 0.377)	p<0.001	N/A	N/A	0.288 (0.213, 0.362)	p<0.001	0.253 (0.198, 0.307)	p<0.001	N/A	N/A
1 month	0.113 (0.057, 0.170)	p<0.001	N/A	N/A	0.131 (0.062, 0.201)	p<0.001	0.137 (0.068, 0.206)	p<0.001	N/A	N/A
2 months	0.119 (0.058, 0.180)	p<0.001	N/A	N/A	0.146 (0.062, 0.229)	p=0.001	0.113 (0.044, 0.182)	p=0.001	N/A	N/A
3 months	0.095 (0.038, 0.152)	p=0.001	N/A	N/A	0.102 (0.035, 0.169)	p=0.003	0.088 (0.023, 0.154)	p=0.008	N/A	N/A
4 months	0.099 (0.036, 0.161)	p=0.002	N/A	N/A	0.102 (0.024, 0.179)	p=0.01	0.083 (0.016, 0.150)	p=0.01	N/A	N/A
6 months	0.082 (0.029, 0.136)	p=0.002	N/A	N/A	0.072 (0.015, 0.129)	p=0.01	0.046 (-0.008, 0.100)	p=0.10	N/A	N/A
11 months	0.061 (0.005, 0.117)	p=0.03	N/A	N/A	0.078 (0.023, 0.134)	p=0.006	0.061 (0.005, 0.117)	p=0.03	N/A	N/A
14 months	0.080 (0.012, 0.148)	p=0.02	N/A	N/A	0.065 (0.007, 0.112)	p=0.03	0.042 (-0.015, 0.099)	p=0.14	N/A	N/A
24 months	N/A	N/A	N/A	N/A	0.055 (-0.011, 0.120)	p=0.10	0.039 (-0.026, 0.105)	p=0.23	N/A	N/A

Values are regression coefficients (95% confidence interval) and their p-values and represent the change (standard deviation score (SDS)) of head circumference, biparietal diameter, (femur) length, (estimated fetal) weight and abdominal circumference per increase of 1 standard deviation in first-trimester fetal crown to rump length. All models were adjusted for fetal sex.

References

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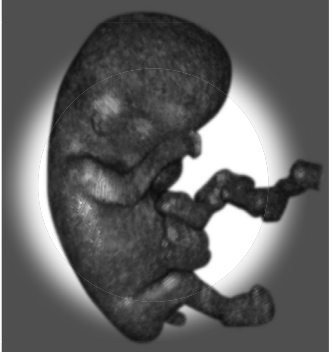
2. Rigby RA, Stasinopoulos DM. Generalized Additive Models for Location, Scale and Shape. *Applied Statistics*. 2005;54:507-554.
3. *SAS/STAT 9.2 User's Guide: The MIXED Procedure* Cary, North Carolina, U.S.A.: SAS Publishing; 2009.



CHAPTER 2.2

Heritability of body size from fetal life to early childhood

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Submitted

Abstract

Background: We estimated the heritability for height and weight during fetal life and early childhood.

Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards. Parental height and weight were measured and fetal growth characteristics (femur length and estimated fetal weight) were assessed by ultrasounds in 2nd and 3rd trimester and at birth (length and weight). Postnatally, height and weight were assessed at eight time-points between 1 and 36 months. Heritability was estimated using regression models as proposed by Galton.

Results: Heritability estimates for height increased between 2nd and 3rd trimester from 13% (95% CI: 8%, 17%) to 28% (95% CI: 24%, 33%). In the first month of postnatal life, height heritability estimates increased rapidly from 26% at birth (95% CI: 21%, 32%) to 41% (95% CI: 36%, 46%), followed by a gradual increase to 63% (95% CI: 58%, 68%) at 36 months. Heritability estimates for weight increased between 2nd and 3rd trimester from 17% (95% CI: 12%, 21%) to 27% (95% CI: 23%, 31%), and gradually from 26% (95% CI: 22%, 31%) at birth to 42% (95% CI: 37%, 48%) at 36 months.

Conclusions: Heritability estimates of height and weight increase from second trimester to infancy. The estimates were low during the first half of pregnancy and slightly higher in third trimester. After birth, heritability estimates for height were consistently higher than for weight.

Introduction

Heritability is the proportion of variability of a phenotype which can be explained by shared genes and environment. Total adult body height is considered to be a highly heritable trait, with an estimated heritability of about 90%.[1] Generally, the heritability of weight and body mass index is considered to be lower with estimates ranging from 16% to 85%.[2] Heritability is usually estimated through twin studies, where correlation coefficients of monozygotic and dizygotic can be compared.[1,3] Alternatively, Galton suggested that the heritability of height can also be estimated by regressing the height of offspring against the mid-parental height.[4] This method has been shown to have a high predictive accuracy for the height of the offspring.[5] Cole demonstrated that the accuracy of this method could further be improved by using standard deviation scores instead of height measurements in centimetres.[6]

Heritability estimates on anthropometrics are often obtained from single cross-sectional measurements.[1,7-11] Few studies focused on heritability of anthropometrics throughout early, especially fetal, life.[12-16] As compared to final adult height and weight, the heritability of size at birth is low.[14,15,17] In a parent-offspring cohort amongst more than 100,000 families, the fetal genetic contribution to birth weight was suggested to be around 31%, while for birth length it was about 27%.[17] Maternal genetic factors accounted for 22% and 19% of the variance in birth weight and length, respectively.[17] In a twin birth cohort, it was suggested that the heritability of birth weight between 25 and 42 weeks of gestation decreased from 52% to 30%.[14] This decrease could be explained by the fact that the shared maternal-uterine environment becomes more dominant than genetic factors in third trimester fetal growth. These findings also suggest that the genetic contribution to growth should increase from birth to adulthood.

For the present study, we hypothesized that the heritability of weight and height would be relatively high during the first half of pregnancy, lower during third trimester or at birth and gradually increase throughout early childhood. We tested this hypothesis in a population-based prospective cohort study from early fetal life onwards among 3,407 Caucasian children and their parents. We estimated the heritability for height and weight during fetal life and early childhood using the models proposed by Galton and by Cole.[4,6]

Methods

Study design and cohort

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards. This study is designed to identify early determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[18,19] Enrollment was aimed for in first trimester but was allowed until birth. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Population for analysis

Analyses were restricted to parent-child trio's of singleton pregnancies from Dutch or other European Caucasian ethnicity with parental height and weight data available (n = 3,407). Due to miscarriage and prenatal loss to follow-up, data at birth was collected for 3,370 newborns. The prenatal follow-up rate was 95%. For the postnatal analyses, 9% of the study population lived outside the study area, leaving 3,084 subjects. The postnatal overall follow-up rate was 73%.

Parental anthropometrics

Maternal pre-pregnancy weight was obtained through questionnaire at the enrolment in the study. Since enrolment in our study was in early pregnancy, we were not able to measure maternal weight before pregnancy. Correlation of pre-pregnancy weight obtained by questionnaire and weight measured at enrolment (median gestational age 13.5 (90% range 10.8 – 21.4) was 0.97 ($P < 0.001$). Maternal height and paternal weight and height were measured at our research center using standardized procedures. Height was measured to the nearest millimeter using a standiometer (Holtain Ltd., Dyfed, U.K.) and weight was measured to the nearest 0.1 kg using electronic scales (SECA, Hamburg, Germany).

Fetal growth measurements

Fetal ultrasound examinations were carried out at the research centers in each trimester of pregnancy.[20,21] These fetal ultrasound examinations were used for both establishing gestational age and assessing fetal growth characteristics. Crown-rump length was used for pregnancy dating in early pregnancy (gestational age until 12 weeks and 5 days,

crown-rump length smaller than 65 mm) and biparietal diameter was used for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, biparietal diameter larger than 20 mm). For the present study, we measured fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) to the nearest millimeter using standardized ultrasound procedures in second and third trimester (median ages: 20.5 weeks (90% range: 19.0 – 22.6) and 30.4 weeks (90% range: 28.9 – 32.4), respectively).[21] Estimated fetal weight (EFW) was calculated using the formula by Hadlock ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 (HC)^2 – 0.003685 (AC * FL)).[22] Ultrasound examinations were performed using an Aloka® model SSD-1700 (Tokyo, Japan) or the ATL-Philips® Model HDI 5000 (Seattle, WA, USA). Fetal measurements in early pregnancy were not included as growth characteristics because these ultrasound examinations were primarily performed to establish gestational age.

Childhood growth measurements

Date of birth, birth anthropometrics (length and weight) and offspring sex were obtained from community midwife and hospital registries. Well-trained staff in community health centers obtained postnatal growth characteristics (length and weight) using standardized procedures.[18] Based on the routine health care program, visits for these growth characteristics were grouped into eight age periods. Median (90% range) ages (in months) of these periods were: 1.1 (0.9 – 1.6); 2.2 (2.0 – 2.9); 3.3 (3.1 – 3.9); 4.4 (4.0 – 4.9); 6.1 (5.4 – 7.3); 14.2 (13.6 – 15.9); 24.7 (23.4 – 27.3); and 36.5 (36.5 – 39.5).

Statistical analysis

Standard deviation scores

We created study-based standard deviation scores (SDS) for all growth characteristics. These scores enable adjustment for (gestational) age avoiding the inclusion of non-linear functions of (gestational) age in models. Our approach for developing reference models for constructing SDS was based on the LMS model of Cole and Green,[23] as implemented in the GAMLSS software of Rigby and Stasinopoulos.[24] The LMS model of Cole and Green assumes that, after a Box-Cox transformation, the growth characteristic has a normal distribution. The acronym LMS stands for lambda, mu, and sigma. Mean and standard deviation of the distribution are allowed to change with (gestational) age, possibly by smooth curves, described by polynomials of splines. The formula for creating standard deviation scores is:

$$z = \frac{(y/\mu(x))^{\lambda(x)} - 1}{\lambda(x)\sigma(x)},$$

where x stands for (gestational) age and y for the growth parameter. The model comprises three curves: λ(x) for the parameter of the Box-Cox transformation, μ(x) for the mean of the transformed height or weight and σ(x) for its standard deviation. Once the curves λ(x), μ(x) and σ(x) have been obtained, one can fill in any data pair (x, y) and obtain the standard deviation score z. The curves in the LMS can be flexible, but they might also be straight lines or even constants depending on the data. The GAMLSS software of Rigby and Stasinopoulos allows one to experiment with a large number of settings and to judge the quality of models by means of the log-likelihood and Akaike's information criterion (AIC).

Heritability estimates

First, mid-parental height and weight standard deviation score (SDS) was created by taking the average of the two parents. Subsequently, the heritability estimate (h^2) was determined using the method of Galton.[4] The slope of the regression line (β1) approximates the heritability when the trait of the offspring is regressed against the average trait in the parents. Since fetal body length can not be measured, femur length in second and third trimester was used as a proxy for body length prenatally.[25] Finally, to distinguish between paternal and maternal heritability, we regressed the height of the child against both paternal and maternal separately using a single parent-offspring model. When the offspring growth characteristic is regressed against a single parent, the heritability estimates (h^2) is equal to regression line (β1) multiplied by a factor of two.[4] Similar models were used for weight. Statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA) and R version 2.10.1 (The R Foundation for Statistical Computing).

Results

Table 1 and Table 2 give the parental, fetal and childhood anthropometric characteristics. Figure 1 show the heritability estimated for height and weight from second trimester to 36 months. For both height the heritability increased strongly between second trimester and third trimester (12.6% (95% confidence interval (CI): 8.0%, 17.1%) to 28.1% (95% CI: 23.7%, 32.5%), after which we observed a slight decrease in heritability estimates during third trimester. In the first month of life, the heritability of height increased rapidly from 26.4% at birth (95% CI: 21.2%, 31.6%) to 40.9% (95% CI: 35.5%, 46.2%). This increase was followed by a more gradual increase to a heritability of 63.4% (95% CI: 58.3%, 68.4%) at 36 months. The heritability for weight had a similar pattern for fetal growth to height increasing strongly between second and third trimester (from 16.6% (95% CI: 12.1%, 21.1%) to 27.1% (95% CI: 22.9%, 31.4%)), followed by a slight decrease in third trimester.

Table 1: Parental characteristics

Parental characteristics	N	Mean (SD) / Median (90% range)
Maternal characteristics		
Age (years)	3407	31.5 (4.1)
Height (cm)	3405	170.6 (6.4)
Standard deviation score	3405	0.00 (1.00)
Weight (kg)	3394	68.0 (55.0, 94.0)
Standard deviation score	3394	-0.09 (-1.24, 1.90)
Paternal characteristic		
Age (years)	3406	33.6 (4.9)
Height (cm)	3407	184.3 (7.0)
Standard deviation score	3407	0.00 (1.00)
Weight (kg)	3401	84.5 (67.0, 108.0)
Standard deviation score	3401	-0.08 (-1.46, 1.79)
Mid-parental characteristics		
Height (cm)	3404	177.4 (5.2)
Standard deviation score	3404	0.00 (0.78)
Weight (kg)	3388	77.0 (64.0, 95.5)
Standard deviation score	3388	-0.08 (-1.11, 1.40)

Values represent means (standard deviation) or median (90% range).

Table 2: Child characteristics

Child characteristic	N	Mean (SD)
Sex (% boys)	3370	50.3%
Second trimester		
Gestational age (weeks)	3171	20.5 (19.0 – 22.6)
Femur length (mm)	3171	33.3 (3.3)
Estimated fetal weight (grams)	3154	380 (87)
Third trimester		
Gestational age (weeks)	3248	30.4 (28.9 – 32.4)
Femur length (mm)	3203	57.5 (3.0)
Estimated fetal weight (grams)	3234	1638 (260)
Birth		
Gestational age (weeks)	3267	40.1 (36.7 – 42.1)
Length (cm)	2282	50.5 (2.3)
Weight (grams)	3253	3514 (509)
1 month		
Age (months)	2409	1.1 (0.9 – 1.6)
Length (cm)	2026	54.5 (2.4)
Weight (grams)	2407	4454 (619)

Table 2: Continued

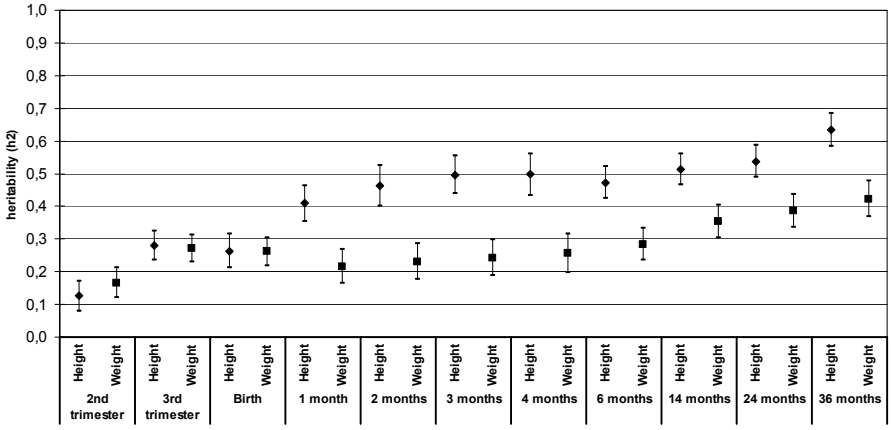
Child characteristic	N	Mean (SD)
2 months		
Age (months)	2084	2.2 (2.0 – 2.9)
Length (cm)	1501	58.6 (2.7)
Weight (grams)	2081	5540 (748)
3 months		
Age (months)	2112	3.3 (3.1 – 3.9)
Length (cm)	1777	61.6 (2.5)
Weight (grams)	2109	6271 (763)
4 months		
Age (months)	1897	4.4 (4.0 – 4.9)
Length (cm)	1398	64.2 (2.5)
Weight (grams)	1892	6906 (788)
6 months		
Age (months)	2698	6.1 (5.4 – 7.3)
Length (cm)	2394	67.7 (2.6)
Weight (grams)	2686	7814 (857)
14 months		
Age (months)	2520	14.2 (13.6 – 15.9)
Height (cm)	2503	78.3 (2.7)
Weight (grams)	2502	10527 (1089)
24 months		
Age (months)	2276	24.7 (23.4 – 27.3)
Height (cm)	2240	88.3 (3.4)
Weight (grams)	2272	12924 (1422)
36 months		
Age (months)	2063	36.5 (35.3 – 39.5)
Height (cm)	2029	97.5 (3.8)
Weight (grams)	2040	15223 (1728)

Values represent means (standard deviation) or percentages.

After birth, the heritability for weight showed a slight decline followed by a gradual increase to a heritability of 42.3% (95% CI: 36.8%, 47.7%) at 36 months.

The heritability of height and weight based on each parent separately is shown in Table 3. For height, the heritability estimates based on maternal height were consistently higher as compared to those based on paternal height from second trimester to 3 years of age. The heritability estimates for second and third trimester estimated fetal weight, based on maternal weight were higher than those based on paternal weight (second trimester: 26.2% (95% CI: 19.2%, 33.2%) for maternal weight versus 14.0% (95%

Figure 1: Heritability of height and weight from second trimester of pregnancy until the age of 36 months.



Values reflect heritability estimates (95% confidence interval)
*Model: height or weight (SDS) = β0 + β1 * mid-parental height or weight (SDS).*
Here the slope 'β1' is equal to the heritability 'h²'.[4]
Prenatally height is femur length (SDS) and weight is estimated fetal weight (SDS)

Table 3: Heritability of height and weight from second trimester of pregnancy until the age of 36 months stratified by parent.

Age	Estimated height heritability (h2)		Estimated weight heritability (h2)	
	Based on mother	Based on Father	Based on Mother	Based on Father
Second trimester	0.194 (0.124, 0.264)	0.106 (0.036, 0.174)	0.262 (0.192, 0.332)	0.140 (0.070, 0.210)
Third trimester	0.392 (0.322, 0.460)	0.280 (0.212, 0.348)	0.430 (0.362, 0.496)	0.232 (0.164, 0.300)
Birth	0.356 (0.274, 0.438)	0.278 (0.196, 0.358)	0.436 (0.368, 0.504)	0.208 (0.138, 0.276)
1 month	0.550 (0.466, 0.632)	0.434 (0.350, 0.518)	0.280 (0.198, 0.362)	0.252 (0.172, 0.330)
2 months	0.662 (0.566, 0.758)	0.440 (0.340, 0.538)	0.284 (0.196, 0.372)	0.288 (0.202, 0.374)
3 months	0.684 (0.596, 0.774)	0.488 (0.398, 0.578)	0.262 (0.176, 0.348)	0.332 (0.246, 0.416)
4 months	0.648 (0.548, 0.750)	0.530 (0.430, 0.630)	0.276 (0.184, 0.370)	0.354 (0.264, 0.444)
6 months	0.650 (0.574, 0.726)	0.474 (0.396, 0.552)	0.364 (0.286, 0.440)	0.340 (0.266, 0.416)
14 months	0.630 (0.556, 0.706)	0.574 (0.498, 0.650)	0.430 (0.348, 0.510)	0.436 (0.360, 0.514)
24 months	0.676 (0.598, 0.754)	0.616 (0.536, 0.694)	0.486 (0.404, 0.568)	0.456 (0.376, 0.538)
36 months	0.784 (0.702, 0.866)	0.704 (0.624, 0.786)	0.546 (0.460, 0.632)	0.480 (0.394, 0.566)

Values reflect heritability estimates (95% confidence interval)
*Model for mothers: height or weight (SDS) = β0 + β1 * maternal height or weight (SDS)*
*Model for fathers: height or weight (SDS) = β0 + β1 * paternal height or weight (SDS).*
*The heritability estimates 'h²' based are equal to 2*β1 for single parent-offspring heritability estimates.*[4]
Prenatally height is femur length (SDS) and weight is estimated fetal weight (SDS)

CI: 7.0%, 21.0%) for paternal weight; and third trimester: 43.0% (95% CI: 36.2%, 49.6%) for maternal weight versus 23.2% (95% CI: 16.4%, 30.0%) for paternal weight). The weight heritability estimates based on maternal weight decreased after birth (43.6% (95% CI: 36.8%, 50.4%) at birth to 28.0% (95% CI: 19.8%, 36.2%) at 1 month), after which there were no large differences in weight heritability estimates between the parents until the age of 3 years.

Discussion

In this study we estimated the heritability of body size from second trimester until the postnatal age of 36 months using the regression method of Galton. During this period, the heritability for height and weight increased from 12.6% to 63.4% and from 16.6% to 42.3%, respectively. The heritability of height measures was consistently higher for estimates based on mother's height than on father's height. For fetal weight, the heritability estimates were higher for estimates based on mother's weight than for father's weight, whereas for childhood weight, the heritability estimates were similar for those based on maternal or paternal weight.

Prior to the study, we had hypothesized that the heritability of height and weight would be relatively high in the first half of pregnancy, lower in third trimester or birth and gradually increase throughout early childhood. However, we observed that the heritability estimates for height and weight at second trimester were considerably lower in second trimester than third trimester. These low estimates could be the result of the fact that fetal growth is thought to be fairly uniform during early pregnancy and that therefore the variance of the measurements is too low. However, another possible explanations might be that the measurement error is too high in second trimester, leading to a low estimated heritability. Furthermore, the standard deviation scores in our study take gestational age into account as determined by crown-rump length in early pregnancy (< 15 weeks of gestation). We have previously shown that gestational age based on crown-rump length can deviate from gestational age based on the first day of last of menstrual period.[26] Using gestational age based on crown-rump length could lead to random misclassification of the standard deviation scores and therefore an underestimation of heritability estimates. However, the heritability estimates were similar in a subset of the individuals with gestational age based on first day of last menstrual period to those based on crown-rump length (data not shown).

A previous study demonstrated that heritability of birth weight decreased between 25 and 42 weeks of gestation from 52% to 30%.[14] In our study, the heritability of birth weight was slightly lower (26.2%) and using directly measured fetal growth characteristics, we showed that the heritability slightly decreased between during the last ten

weeks of pregnancy. This decrease could be explained by a more dominant role for the uterine environment in third trimester fetal growth. At the age of 36 months the heritability for height and weight was estimated to be 63.4% and 42.3%, respectively. These estimates are lower than those estimated in adulthood.[1,2] We expect that these values would increase further into adulthood. This increase in heritability might be expected to occur before or during puberty, since the onset of the pubertal growth spurt and the age of peak height velocity have been shown to be highly heritable (91% and 93%, respectively).[11] The estimated heritability for postnatal length was consistently higher for maternal height than for paternal height. An explanation might be that mothers have a larger shared postnatal environment with their offspring than fathers in early life. However, this does not explain why we did not observe a difference between the two parents regarding the heritability of weight in their offspring. Another explanation might be that not all fathers in our study are the biological father, which would of course lead to smaller heritability estimates based on father, due to the decrease in shared genes. Finally, a parent-of-origin effect on early growth of genes regulating growth, as is known in the case of the genomic imprinting of the *IGF2* gene,[27] might explain part of the difference in heritability estimates between the parents.

Height heritability estimates increased strongly during the first month postnatally, while the increase for weight was more gradual. Studies have shown that children tend to catch-up or catch-down in the first years after birth, after which growth generally continues along the same percentile until the individual reaches the target height in adulthood.[28] Changes in early postnatal growth rates are influenced by a drive to compensate for prenatal fetal growth restriction or growth enhancement caused by the maternal-uterine environment.[28] The heritability of postnatal weight gain has been estimated to be relatively high at around 80%.[15] Our study would indicate that a significant proportion of this catch-up or catch-down growth in height would occur already in the first few weeks of postnatal life and that this compensation takes longer for weight.

Some methodological issues need to be considered. To our knowledge, this is the first study that longitudinally assessed the heritability of body size from fetal life onwards. Most studies estimated heritability on twins, which can be problematic when assessing fetal growth since these growth patterns are quite different from singleton pregnancies.[29] The regression method from Galton,[4] modified by Cole,[6] allowed us to assess heritability of growth using parent-offspring trios. However, this method might lead to an overestimation of the genetic contribution in utero. For example, we observed a relatively high heritability estimate for fetal weight and birth weight based on maternal weight as compared to paternal weight. Postnatally, the parental contribution to the heritability of weight was similar between the two parents. Maternal pre-pregnancy weight is known to be highly positively associated with fetal growth.[30] The heritability estimate we used is not only a reflection of shared genes, but also shared environment.

Therefore, the relatively higher fetal weight heritability estimates for maternal weight than for paternal weight is most likely a reflection of a shared maternal-fetal environment rather than shared genetic factors. Finally, in our study the postnatal follow-up rate was 73%. It is unlikely, however, that this loss to follow-up biased our results, since one can assume that the follow-up is independent of the heritability.

In conclusion, the current study demonstrated that the heritability of height and weight increases from second trimester to infancy. After birth, heritability estimates for height were consistently higher than weight. Longer follow-up studies are necessary to examine how the heritability develops in later childhood and puberty.

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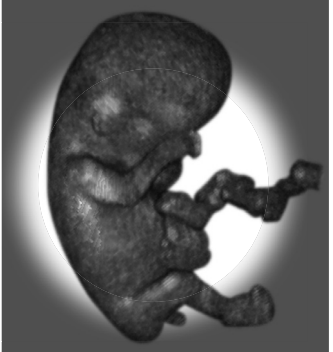

CHAPTER 2.3

Abdominal fat in children measured by ultrasound and computed tomography

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Abstract

Background: The prevalence of childhood obesity is rapidly increasing. Visceral fat has an important role in the pathogenesis of metabolic and cardiovascular diseases. Currently, Computed Tomography (CT) is broadly seen as the most accurate method of determining visceral fat. The main objective was to examine whether measures of abdominal visceral fat can be determined by ultrasound in children and whether CT can be replaced by US for this purpose.

Methods and Results: To assess whether preperitoneal fat thickness and area are a good approximation of visceral fat at the umbilical level, we first retrospectively examined 47 CT-scans of non-obese children (BMI under 30 kg/m^2) (median age 7.9 (95% range: 1.2 – 16.2) years). Correlation coefficients between visceral and preperitoneal fat thickness and area were 0.58 ($p<0.001$) and 0.76 ($p<0.001$), respectively. Then, to assess how preperitoneal and subcutaneous fat thicknesses and areas measured by ultrasound compare to these parameters in CT, we examined 34 non-obese children (median age 9.5 (95% range: 0.3 – 17.0) by ultrasound and CT. Ultrasound measurements of preperitoneal and subcutaneous fat were correlated with CT measurements, with correlation coefficients ranging from 0.75 to 0.97 (all $p<0.001$). Systematic differences of up to 24.0 cm^2 for preperitoneal fat area (95% confidence interval: -29.9 to 77.9 cm^2) were observed when analyzing the results described by the method of Bland and Altman.

Conclusions: Our findings suggest that preperitoneal fat can be used as an approximation for visceral fat in children and that measuring abdominal fat with ultrasound in children is a valid method for epidemiological and clinical studies. However, the exact agreement between the ultrasound and CT-scan was limited, which indicates that ultrasound should be used carefully for obtaining exact fat distribution measurements in individual children.

Introduction

The prevalence of childhood obesity is rapidly increasing and is currently a major health problem worldwide.[1,2] The prevalence in the United States increased from 10% to 13% between 2000 and 2004.[2] Also the rate of annual increase has risen from 0.1% in the 1980's to 0.3% in the late 1990's.[3] Childhood obesity is an important risk factor for developing metabolic syndrome in adulthood, including type 2 diabetes, and cardiovascular diseases.[4] Epidemiological studies have shown that rather than high body mass index, visceral fat has an important role in the pathogenesis of several metabolic and cardiovascular diseases both in adults and children.[5-8] The ratio between visceral and subcutaneous fat is significantly associated with several metabolic risk factors.[9,10] Visceral fat has been demonstrated to be correlated to insulin, cholesterol and triglyceride levels in children.[7,11] Furthermore, several plasma markers of adiposity such as interleukin-6 and adiponectin levels have been shown to be associated specifically with the amount of visceral fat.[12] Therefore, rather than studying body mass index and obesity, we expect that studies focused on abdominal fat distribution in children will lead to better insight in the causes of adverse body composition and the consequences for development of metabolic and cardiovascular diseases.

Currently, computed tomography (CT) is considered as a valid method to measure abdominal fat distribution.[13-15] CT-scanning cannot be performed easily in healthy children due to radiation exposure and is therefore unsuitable for epidemiological or clinical research purposes. Magnetic Resonance (MR) imaging, which also allows very detailed information on fat distribution, is radiation free but is time consuming, strenuous for the child and expensive.[16] Alternatively, ultrasound is also radiation free and easy to perform in children. With ultrasound, preperitoneal fat can be measured which is known to be an appropriate proxy for visceral fat in adults.[17] However, these results cannot be easily extrapolated to young children.

Therefore, we set out to examine whether measures of abdominal visceral fat can be determined by ultrasound in children and whether CT can be replaced by ultrasound for this purpose. We first examined whether preperitoneal fat is a good approximation for visceral fat in children using within CT-scan comparisons. Then, we compared measures of subcutaneous and preperitoneal fat in children using abdominal ultrasound and CT methodology.

Materials and Methods

Comparison visceral fat and preperitoneal in CT-scans (Sub-study 1)

Design and subjects

All abdominal CT-scans performed at the Radiology department of the Erasmus Medical Center – Sophia Children's Hospital, a university hospital in Rotterdam, the Netherlands, between January 2003 and December 2007 were retrospectively evaluated (n=424). All CT-scans were performed for medical reasons. For this study, the liver needed to be fully visible to calculate preperitoneal fat. Of these CT-scans, 171 were excluded due to insufficient scan quality or an insufficient field of view, 113 were excluded due to abdominal anatomical abnormalities, such as ascitis or abdominal tumors, 53 measurements were excluded due to repeated CT-scans of the same individual, 35 were excluded due to the age above 18 years and 5 were excluded due to obesity (BMI above 30 kg/m^2). In total, 47 CT-scans remained for analysis (29 boys, 18 girls, median age (95% range): 7.9 (1.2 – 16.2) years).

CT-scanning measurements

All CT-scans were performed on a multidetector 6 slice (Siemens Somatoscan, Erlangen, Germany) in supine position. A pediatric radiologist determined the protocol used for scanning, including the field of view. To facilitate the procedure of measuring the thickness and area in CT data, we used an in-house developed semi-automatic drawing and measuring tool using MevisLab (Bremen, Germany) as software environment.[18] This tool directly imports CT data, has various visualization and image interpolation options, and supports the drawing of lines and closed contours on e.g. the transversal and sagittal planes. All measurements were performed off-line, using linear interpolation, with a fixed window width and window level settings of 600 HU (Hounsfield Units) and 100 HU, respectively. All CT measurements were performed by the same (LMH) research assistant.

Intra-abdominal visceral fat was determined in a transversal slice at the position of the umbilicus. A fixed range of HU was used to determine the fat area. Pixels with HU between –190 and –30 were defined as fat.[19] All area measurements were performed in a sagittal plane at the level of the umbilicus. The abdominal muscle wall was used as a contour for the intra-abdominal tissue. The visceral fat area was determined by calculating the area of all pixels with an attenuation coefficient between -190 and –30 HU within the contour for the intra-abdominal tissue. The total abdominal fat area was determined by calculating the area of all pixels with an attenuation coefficient between -190 and -30 HU in the entire sagittal plane. The subcutaneous fat area was calculated by subtracting

the visceral fat area from the total abdominal fat area. Then, the ratio was determined between visceral and subcutaneous fat area.

Subsequently, the position of the maximum preperitoneal fat thickness was established. The maximum preperitoneal fat thickness is located at the upper part of the ventral side of the liver. In a transversal slice the maximum preperitoneal fat thickness was measured (Figure 1A). The thickness of the subcutaneous fat layer was measured at the same position. Then, an area of preperitoneal and subcutaneous fat was measured in a sagittal plane starting at the maximum preperitoneal fat thickness and measuring 20 mm in the caudal direction (Figure 1B). Finally, preperitoneal-to-subcutaneous ratios could be calculated for both fat thickness and area.

Data Analysis

To evaluate associations between visceral fat and preperitoneal fat, we calculated Pearson's correlation coefficients (*r*). We compared visceral fat to both preperitoneal transversal thickness and preperitoneal area. We also calculated correlation coefficients between the various subcutaneous fat measurements and between visceral/subcutaneous fat ratio and preperitoneal/subcutaneous fat ratio.

Comparison preperitoneal and subcutaneous fat measured by ultrasound and CT (Sub-study 2)

Design and subjects

The subjects were prospectively selected from the Radiology department of the Erasmus Medical Center - Sophia Children's Hospital. Each subject who was planned for a CT examination of the thorax or abdomen for medical reasons was approached for this study. Subjects with substantial abdominal anatomical abnormalities (ascitis or abdominal tumors) were excluded. CT scanning and ultrasound examination were performed on the same day. All subjects (or their parents) gave informed consent for participating in this study. We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. The study was approved by the medical ethics committee of the Erasmus Medical Center.

Children were enrolled between January 2008 and June 2008. Only outpatients were eligible for participation in this study. We approached 52 children to participate in the study. Ten patients could not be included due to either no consent or logistical constrains (e.g. patient too ill to participate). In total, 41 children participated in this validation study. Of all 41 CT-scans that were performed, 6 were abdominal scans and 35 were thoracic scans. We performed 40 ultrasound examinations. One ultrasound examination could not be performed due to crying behaviour of the patient. We excluded 6 children from the analyses due to a too small field of view of the CT-scan, which left 34 subjects

(31 thoracic, 3 abdominal; 17 boys, 17 girls, median age (95% range): 9.5 (0.3 – 17.0) years) for final analysis.

Anthropometrics

Anthropometrics of the children were measured lightly clothed and without shoes. Weight was measured to the nearest 0.1 kg by an electronic personal scale (SECA 888). Height was measured in standing position to the nearest 0.1 cm by a Harpenden stadiometer (Holtain Limited), except for one subject (age 3 months), where height was measured in the supine position. Body Mass Index (BMI) was calculated (kg/m^2).

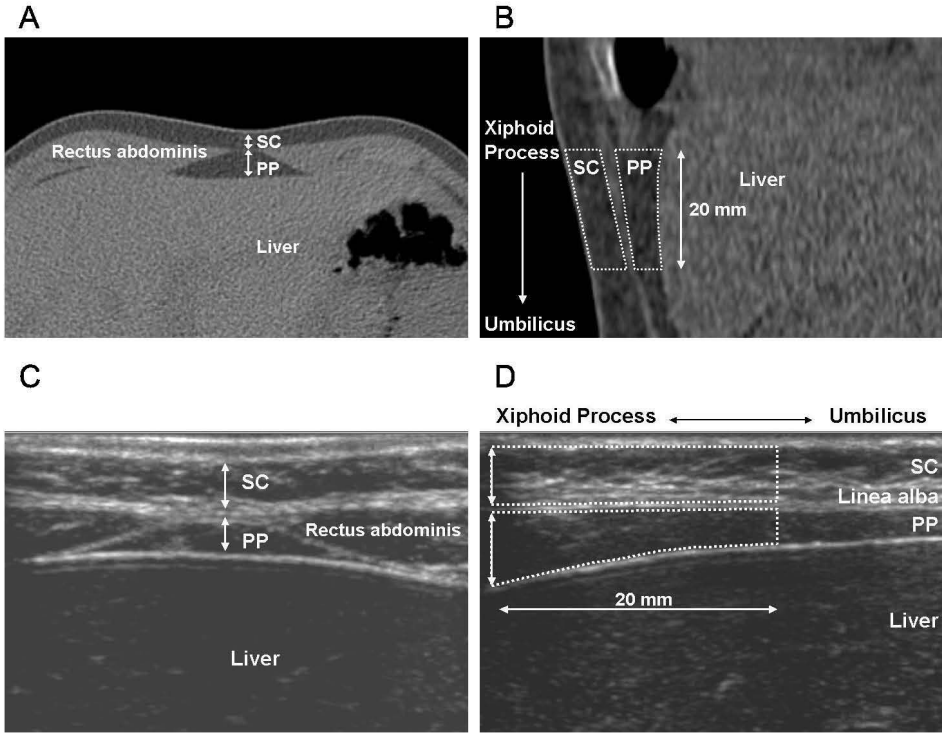
CT-scanning and ultrasound measurements

All CT measurements were performed using the same method as in sub-study 1. Ultrasound measurements were performed with the Philips / ATL HDI 5000 (Best, the Netherlands) in the supine position. Preperitoneal and subcutaneous fat thickness and area were measured with a linear array probe L12-5 (38 mm, 5-12 MHz), according to the method of Suzuki.[17] The linear probe was carefully positioned perpendicular to the skin of the median upper abdomen and touched as lightly as possible to prevent compression of the fat layers. Scanning was performed while moving the probe longitudinally from the xiphoid process and the umbilicus along the midline to obtain an image which contained the maximum preperitoneal fat thickness. A transversal image was obtained by placing the probe perpendicularly to the linea alba. Only the thicknesses, and not the areas, of the fat layers were measured in this transversal image (Figure 1C). The preperitoneal fat thickness was determined by the maximum height of the triangular shaped area in the transversal image (Figure 1C). The maximum subcutaneous fat thickness was measured directly above this triangular area (Figure 1C).

To obtain a sagittal image, the probe was kept parallel to the linea alba (Figure 1D). In this image, we first determined the maximum preperitoneal thickness which, as in the CT images, is located at the upper part of the ventral side of the liver. At this same level we determined the subcutaneous thickness. Subsequently, we measured the maximum preperitoneal and subcutaneous areas starting from the position of the maximum preperitoneal thickness over a distance of 20 mm in the caudal direction (Figure 1D). The maximum subcutaneous fat area was measured in parallel along the same 20 mm distance. Finally, preperitoneal-to-subcutaneous ratios were determined for both fat thickness and area.

CT-scans were performed with subjects in an extended breath-hold position and ultrasound measurements were performed while subjects breathed regularly. Therefore, we assessed the maximum fat thickness and area between the xiphoid process and the umbilicus in both CT and ultrasound measurements, rather than measuring these parameters at one specific point. Analyses of the ultrasound and CT images were

Figure 1: Measurements of abdominal fat with CT and ultrasound



*Transversal images from a CT-scan (**A**) and an ultrasound (**C**), at the location where the maximum preperitoneal (PP) thickness and the subcutaneous (SC) thickness were measured. Sagittal images from a CT-scan (**B**) and an ultrasound (**D**), where the PP and SC area were measured starting at the location of the maximum PP thickness to 20 mm in caudal direction.*

independently performed. All CT scans were performed by radiology technicians from the pediatric radiology department. Analyses of the CT images were performed by one investigator (LMH) under supervision of the pediatric radiologist (MHL). All ultrasound measurements and off-line image analyses were performed by one ultrasound technician.

Data Analysis

The intra-observer repeatability of both methods was assessed by intra-class correlation coefficients.[20] We calculated Pearson's correlation coefficients (*r*) between CT and ultrasound data for all measurements. To measure the exact agreement between both methods we performed a Bland and Altman analysis.[21,22] We estimated the maximum acceptable difference between the CT-scan and ultrasound to be 2 mm (+/- 1 SD) for fat thickness measures. For the areas this results in an acceptable difference of 40 mm^2 (2 mm over the length of 20 mm). To create Bland and Altman plots, the differences

between the methods are plotted against their mean, in which the basic assumption is made that the differences are normally distributed. Based on these data we calculated the mean difference (M_d) and the SD of the differences (SD_d). The M_d +/- 2 SD_d are referred to as 'the limits of agreement'.

Results

Comparison visceral fat and preperitoneal in CT-scans

All correlation coefficients are shown in Table 1. We observed significant correlations between visceral fat area and preperitoneal fat thickness and area (r = 0.58 ($p<0.001$), and r = 0.76 ($p<0.001$), respectively). Stronger correlations were found for within CT measured subcutaneous fat measurements ($r > 0.82$ ($p<0.001$)). Lower correlations coefficients were found between the ratio of visceral/subcutaneous fat and preperitoneal / subcutaneous fat thickness (r = 0.36, $p<0.05$). Similar results were found for both males and females (data not shown).

Table 1: Abdominal fat correlations measured by Computed Tomography in 47 subjects

		r	p- value
Visceral area	PP thickness transversal	0.58	< 0.001
Visceral area	PP area	0.76	< 0.001
Subcutaneous area	SC thickness transversal	0.83	< 0.001
Subcutaneous area	SC area	0.82	< 0.001
V/S area ratio	PP/SC thicknesses transversal ratio	0.36	< 0.05
V/S area ratio	PP/SC area ratio	0.18	0.37

Values are Pearson correlation coefficients (r) and are based on PP:preperitoneal, SC:subcutaneous, V/S: Visceral/Subcutaneou

Comparison preperitoneal and subcutaneous fat measured by ultrasound and CT.

Subject characteristics and descriptive statistics of the CT and ultrasound measurements are shown in Tables 2 and 3 respectively. To assess the intra-observer repeatability of our measurements we calculated intra-class correlation coefficients (ICC's). For the CT measurements the ICC's were between 0.990 and 0.998. For the ultrasound measurements the ICC's ranged from 0.973 to 0.976, from which we can conclude that our measurements for CT as well as ultrasound were highly reproducible. All CT and ultrasound measurements were highly correlated (Table 4). For preperitoneal fat and subcutaneous measures, correlation coefficients ranged from 0.75 to 0.84 and from

Table 2: Subject characteristics participating in Computed Tomography and ultrasound comparison study

N	34
Age (y)	
Median (95% Range)	9.5 (0.3-17.0)
Gender	
Male	17
Female	17
Type of CT-scan	
Abdominal	3
Thoracic	31
Weight (kg)	
Median (95% Range)	31.5 (4.5 – 67.0)
Length (cm)	
Median (95% Range)	136.4 (54.0 - 189.1)
Body Mass Index (kg/m^2)	
Median (95% Range)	16.9 (12.2 – 26.8)

Table 3: Descriptive statistics of measurements for Computed Tomography and ultrasound comparison study

	Number	Mean	Min	Max
Preperitoneal				
CT preperitoneal thickness transversal (mm)	33	6.3	1.7	11.9
US preperitoneal thickness transversal (mm)	34	4.7	1.2	12.2
US preperitoneal thickness sagittal (mm)	33	4.2	1.3	10.3
CT preperitoneal area (mm^2)	19	115.4	36.2	187.4
US preperitoneal area (mm^2)	33	70.7	18.0	192.0
Subcutaneous				
CT subcutaneous thickness transversal (mm)	33	3.8	0.5	16.2
US subcutaneous thickness transversal (mm)	34	4.7	0.8	18.9
US subcutaneous thickness sagittal (mm)	33	3.9	0.7	21.1
CT subcutaneous area (mm^2)	18	92.3	19.7	328.6
US subcutaneous area (mm^2)	33	82.5	21.0	308.0

CT is computed tomography, US is ultrasound

0.94 to 0.97, respectively. Similar results were found for analysis of all subjects under 8 years (data not shown). The differences between CT and ultrasound measurements were normally distributed. The mean differences and the limits of agreement (M_d +/- 2 SD_d) are shown in Table 5. Systematic differences were observed for all preperitoneal fat layer

Table 4: Abdominal fat correlations measured by Computed Tomography and ultrasound

Computed Tomography	Ultrasound	r	p value
Preperitoneal			
PP thickness transversal	PP thickness transversal	0.75	< 0.001
PP thickness transversal	PP thickness sagittal	0.76	< 0.001
PP area	PP area	0.84	< 0.001
Subcutaneous			
SC thickness transversal	SC thickness transversal	0.97	< 0.001
SC thickness transversal	SC thickness sagittal	0.94	< 0.001
SC area	SC area	0.97	< 0.001
Ratio's			
PP/SC ratio (thicknesses transversal)	PP/SC ratio (thicknessess transversal)	0.86	< 0.001
PP/SC ratio (thicknesses transversal)	PP/SC ratio (thicknesses sagittal)	0.75	< 0.001
PP/SC ratio (areas)	PP/SC ratio (areas)	0.61	< 0.05

Values are Pearson correlation coefficients (r)
PP:preperitoneal, SC:subcutaneous

Table 5: Agreement between by Computed Tomography and ultrasound

				Limits of agreement	
			Mean difference	Lower limit	Upper limit
Computed Tomography	**Ultrasound**				
PP thickness transversal	PP thickness transversal	(mm)	1.8	-1.9	5.5
PP thickness transversal	PP thickness sagittal	(mm)	2.0	-1.5	5.6
PP area	PP area	(mm^2)	24.0	-29.9	77.9
SC thickness transversal	SC thickness transversal	(mm)	-1.1	-3.3	1.1
SC thickness transversal	SC thickness sagittal	(mm)	-0.2	-2.9	2.5
SC area	SC area	(mm^2)	-5.4	-42.8	31.9

Values are based on Bland-Altman plots,
PP is preperitoneal, SC is subcutaneous

measurements and for the subcutaneous thickness in the transversal image. Measuring the preperitoneal fat layer by ultrasound resulted in a smaller maximum thickness and area than measurements performed by CT. Similarly, in the traversal image, the subcutaneous thickness was slightly larger measured by ultrasound than measured by CT.

Discussion

In the first sub-study, we found strong correlations between preperitoneal fat and visceral fat measurements obtained by CT. More importantly, in the second sub-study

we demonstrated that measurements of abdominal fat distribution by ultrasound were highly correlated with abdominal fat distribution measured by CT. Assessment of preperitoneal fat by ultrasound did result in systematic differences compared to the CT-scan assessments. Nonetheless, due to the high correlation between the ultrasound and CT measurements, these results suggest that preperitoneal fat measured by ultrasound can be used as an approximation for visceral fat in children.

Methodological considerations

To our knowledge this study is the first study designed to compare preperitoneal fat with visceral fat in non-obese children. Moreover, no studies have compared ultrasonic assessment of abdominal fat distribution in non-obese children to a well-accepted reference method. Previous studies had already been performed in obese children and adults.[23-25] The strength of our study is that our cohort covers a broad range of ages. Therefore our results are applicable for all periods of childhood with a normal body composition. Ideally, the two sub-studies would have been combined into one validation study. Unfortunately, due to the limited number of eligible abdominal CT-scans (between 5 and 10 per year) we were not able to study the relationship between visceral fat measured by CT and preperitoneal fat measured by ultrasound directly.

The children recruited at the department of Radiology from the Sophia Children's Hospital did not reflect a population of healthy Dutch children, since all subjects were undergoing a CT scan for clinical or medical purposes. Including children in our study who were already planned to undergo a CT-scan prevented extra radiation exposure. For both sub-studies, we excluded several children due to obesity, abdominal anatomical abnormalities, poor quality of the CT, or insufficient field of view. This is not likely to influence the main conclusions of our study because we focused on comparisons of the two measurements within subjects and not on any relation to clinical outcomes in these children.

A potential limitation of a CT-ultrasound comparison study is the limited number of abdominal CT-scans available for analysis. To enlarge the number of participants we also included children who were planned to undergo a CT-scan of the thorax. This enabled us to measure the maximum preperitoneal fat thickness. Due to the limited field of view in the pictures obtained from these children, the preperitoneal and subcutaneous area could not be measured in 16 of the 34 subjects. We completed 18 CT-scans in which we were able to perform area measurements and showed that the correlations coefficients were highly significant (Table 3). Another potential limitation of our study is that the CT-scan was performed during respiratory inspiration and measurements were performed off-line. Ultrasound measurements were performed during free breathing and the maximum preperitoneal fat thickness was directly estimated. Due to subject

movements during ultrasound examination this could have resulted in a different estimation of the maximum preperitoneal fat thickness. This difference would lead to an underestimation of our correlation and agreement analyses.

Ultrasonic assessment of subcutaneous and preperitoneal fat

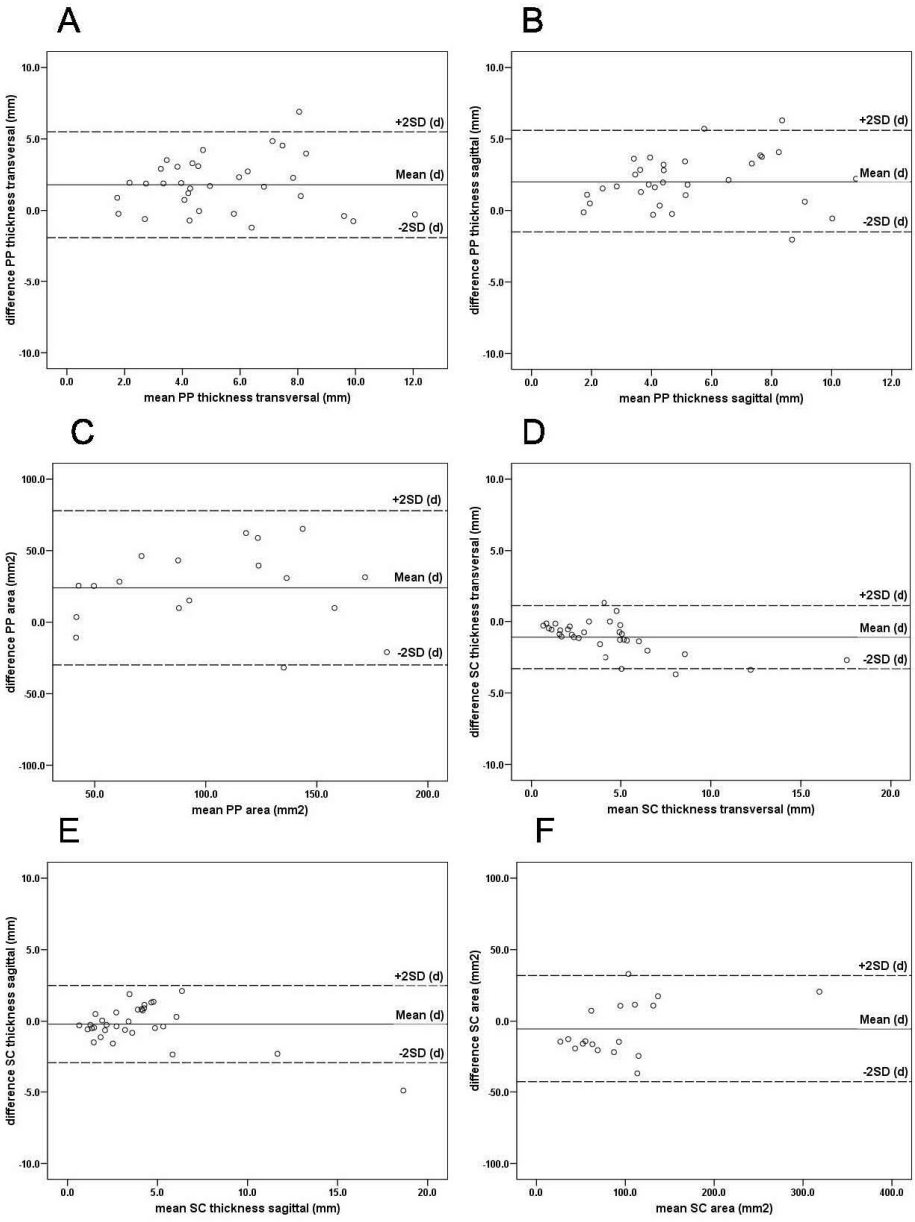
Our results showed high correlations between CT and ultrasound for all measures. Calculating correlation coefficients when comparing different methods can be misleading for clinical applications, because a systematic deviance affects decisions on patient management.[21] However, correlation coefficients do provide useful information for method comparison in epidemiological and clinical research. The high correlation coefficients indicate that the ultrasound method can be used for epidemiological and clinical research to measure abdominal fat distribution.

For the preperitoneal fat layer, the ultrasound measurements were consistently lower than the CT measurements. This systematic difference is probably due to the triangular shape of the preperitoneal fat in non-obese children in a transversal plane (Figure 1A and 1C). The preperitoneal fat layer in normal weight children as well as in adults also decreases in thickness in the caudal direction. A small lateral or caudal shift of the ultrasonic probe could therefore already lead to several millimeters decrease of the estimation of the maximum preperitoneal fat thickness. This effect is multiplied when assessing the preperitoneal area. Furthermore, variations in surface morphology of the liver can make evaluating the preperitoneal fat thickness and area difficult. In contrary, the subcutaneous fat layer in non-obese children has a constant thickness over the whole length and width of the abdomen, which resulted in a better correlation and agreement between ultrasound and CT. Finally, this systematic difference could also be caused by a systematic measurement error in either the ultrasound or the CT. This seems unlikely, but cannot be excluded.

Despite the difficulties to assess the maximum preperitoneal fat thickness with ultrasound, the ratios between the preperitoneal and subcutaneous fat layer (PP/SC ratio) were highly correlated with the PP/SC ratios measured with CT (Table 3). The ratio between the maximum preperitoneal fat thickness and the subcutaneous fat thickness measured at the same position is highly correlated with the ratio between visceral fat and subcutaneous fat measured with CT.[17] Thus, calculating the PP/SC ratio based on ultrasound measures is a useful method to estimate the distribution of abdominal fat.

The exact agreement between the two methods was analyzed using the method described by Bland and Altman.[21,22] We considered a difference of 2 mm below or 2 mm above the mean value of the CT-scan and ultrasound as acceptable. We found that several measurements of the preperitoneal fat thickness did not meet this criterion, while there was better agreement for subcutaneous fat thickness (Table 5). Regarding

Figure 2: Bland and Altman plots



*The Mean (d) indicates the mean difference and the SD (d) indicates the standard deviation of the differences. The mean (d) +/- 2SD (d) are the limits of agreement. A systematic underestimation was observed for the preperitoneal (PP) thickness measured with ultrasound in a transversal image (**A**), PP thickness measured in a sagittal image (**B**) and for PP area (**C**). Ultrasonic assessment of the subcutaneous (SC) thickness in transversal plane resulted in a small overestimation (**D**), which was not the case for SC thickness measured in a sagittal plane (**E**) and SC area (**F**).*

the area measurements, we regarded a maximum difference of 40 mm^2 as acceptable. However, for many of the measurements of the preperitoneal fat the difference in area between CT and ultrasound exceeded this value (Table 5). Therefore, our results suggest that there is a limited level of agreement between the preperitoneal distances and areas measured by CT and ultrasound. This seems to be explained by systematic differences of the measurements by ultrasound. Thus, taking into account this difference using correction formulas, may lead to valid estimates. However, this correction needs to be tested in a larger group of subjects.

Several other methods have been evaluated as a technique to study visceral fat in children and adults, such as skinfold thickness and Dual energy X-Ray absorbtiometry (DXA). Though skinfold thickness is easy and accessible, it is mostly a measure of subcutaneous fat mass. DXA-scans are much more precise and give a good indication of truncal fat mass, but are logistically complicated and more expensive. Furthermore, DXA-scans do not give separate estimates for subcutaneous and preperitoneal fat mass. Our results show that ultrasound is a suitable method for epidemiological and clinical research of abdominal fat distribution in children. The ultrasonic assessment of preperitoneal fat, which we showed to be a good approximation of visceral fat, and subcutaneous fat can be performed within several minutes with minor inconvenience for the child. Ultrasound is radiation free which makes it easy to use in large non-hospital based or disease free populations. Studies are needed to assess determinants and consequences of abdominal fat mass in early childhood. The amount of preperitoneal fat in children could be related to metabolic and cardiovascular outcomes. This may help to predict the risk of the development of adult diseases, such as diabetes type 2 and cardiovascular diseases in obese children.[26,27] Moreover, identification of determinants of accumulation of visceral fat and preperitoneal fat in childhood could lead to prevention of visceral obesity in early life.

Conclusion

In the present study, preperitoneal fat was highly correlated with visceral fat measured by CT. Furthermore, measurements of abdominal fat by ultrasound were strongly correlated with CT but seem to give a small systematic difference. We conclude that measuring abdominal fat distribution using the relatively simple ultrasound methodology is a useful method for epidemiological and clinical research in children but should be used carefully for obtaining absolute measurements in individual children.

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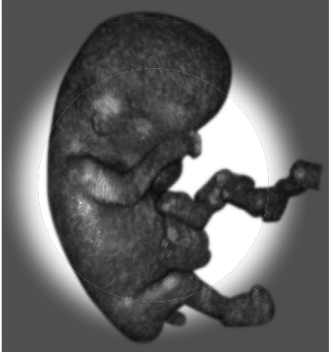

CHAPTER 2.4

Growth in fetal life and infancy as related to abdominal adiposity

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Abstract

Background: Early weight gain is associated with an increased risk of obesity. It is not known whether rapid weight gain in fetal life and infancy is also associated with increased abdominal adiposity. We examined the associations of fetal and postnatal growth characteristics with abdominal fat mass at the age of 2 years.

Methods: This study was performed in 481 children participating in a prospective cohort study from early fetal life onward. Fetal and postnatal growth characteristics in second and third trimester, at birth and at the age of 2 years were related to abdominal fat mass (subcutaneous distance and area, preperitoneal distance and area) measured by ultrasound at the age of 2 years.

Results: Fetal and birth weight were not associated with abdominal subcutaneous fat mass. Estimated fetal weight in second trimester of pregnancy was inversely associated with preperitoneal fat area (-3.73 (95% confidence interval -7.23, -0.10) % per standard deviation score (SDS) increase in weight). Weight gain from birth to the age of 2 years was positively associated with preperitoneal fat mass measures. These associations remained significant after adjustment for age, sex, breastfeeding and body mass index. Positive associations were found between catch up growth in weight and abdominal fat mass measures.

Conclusions: Our results suggest that rapid growth rates during fetal life and infancy are associated with increased abdominal subcutaneous and preperitoneal fat mass in healthy children. Further studies need to explore whether these associations persist in later life and are related to metabolic syndrome outcomes.

Introduction

The prevalence of childhood overweight and obesity has dramatically increased over the past two decades.[1,2] Childhood obesity is an important risk factor for various adverse health outcomes in childhood and adulthood, including type 2 diabetes and cardiovascular diseases.[3,4] Rather than high body mass index, increased central and visceral fat seem to lead to higher risks of development of obesity and metabolic and cardiovascular diseases in later life.[5,6] Studies in adults showed that increased abdominal fat mass, a measure of central and visceral fat, is associated with an increased risk of insulin resistance, dyslipidemia, hypertension and coronary heart disease and overall mortality rates.[7-9] Risk factors for childhood obesity have been studied extensively. Increased growth rates in early postnatal life are strongly associated with obesity in childhood and adulthood.[10,11] It has been suggested that also low birth weight children with an increased postnatal growth rate, are at increased risk for developing obesity.[12] Not much is known specifically about development of abdominal visceral fat in childhood and its growth related determinants.

To test the hypothesis that high growth rates in early life are associated with an increase in abdominal visceral fat mass, which may be related to later health outcomes, we examined the associations of fetal and early postnatal growth characteristics with abdominal subcutaneous and preperitoneal fat mass at the age of 2 years. We also examined whether catch down and catch up growth from birth to the age of 2 years are associated with an increase in abdominal fat mass development. We used a recently developed no-invasive ultrasound method to measure abdominal fat in 481 children participating in a prospective cohort study from early fetal life onwards.[13,14]

Methods

Design

The present study was embedded in the Generation R Study, a prospective cohort study from early fetal life onwards in Rotterdam, the Netherlands. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[13,15] Assessments during pregnancy, including physical and fetal ultrasound examinations and questionnaires, were performed in first trimester (gestational age 14.5 (range 11.2-16.4) weeks), second trimester (gestational age 20.5 (range 19.0 - 22.0) weeks) and third trimester (gestational age 30.3 (range 28.7 - 32.2) weeks). The individual time scheme of these assessments depended on the specific gestational age at

enrolment. At the age of 2 years, abdominal fat measurements were performed in a subgroup of 481 infants. This was a randomly selected subgroup of Dutch children from the total cohort population. No specific selection criteria were used. Of all approached mothers, 80% participated in this study. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents.

Fetal growth

Fetal ultrasound examinations were carried out at the research centers in each trimester of pregnancy.[13,16] These fetal ultrasound examinations were used for both establishing gestational age and assessing fetal growth characteristics. Crown-rump length was used for pregnancy dating in early pregnancy (gestational age until 12 weeks and 5 days, crown-rump length smaller than 65 mm) and biparietal diameter was used for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, biparietal diameter larger than 20 mm). Fetal growth measurements used in the present study included head circumference (HC), abdominal circumference (AC) and femur length (FL), measured in second and third trimester of pregnancy and measured to the nearest mm using standardized ultrasound procedures. Estimated fetal weight (EFW) was calculated using the formula by Hadlock: (log10 EFW=1.5662-0.0108 (HC)+0.0468 (AC)+0.171 (FL)+0.00034 $(HC)^2$-0.003685 (AC x FL)).[17] Fetal measurements in early pregnancy were not included as growth characteristics because these ultrasound examinations were primarily performed to establish gestational age.

Birth characteristics

Date of birth, birth weight (SDS) and gender were obtained from midwife and hospital registries. An decrease and increase in SD score for weight between second trimester of pregnancy and the age of 2 years and between birth and the age of 2 years, greater than 0.67 SD scores were considered as catch-down and catch-up growth, respectively. A change of 0.67 SD represent the width of each percentile band on standard growth charts.[18]

Abdominal fat mass

Abdominal fat mass measures were measured at the age of 2 years with ultrasound (Philips / ATL HDI 5000, Seattle, Washington, USA) in the supine position. A Linear Array probe L12-5 (38 mm, 5-12 MHz) was placed at the median upper abdomen, according to the method described by Suzuki.[14] Scanning was performed between the xiphoid

process and the umbilicus. A transversal image was obtained by placing the probe perpendicularly to the linea alba. Only the maximum thickness of the subcutaneous fat layer was measured in this image by scanning the direction of the xiphoid process (Figure 1A). To obtain a sagittal image, the probe was kept parallel to the linea alba (Figure 1B). In this image the maximum preperitoneal thickness and the subcutaneous thickness were measured. In the same image preperitoneal and subcutaneous areas were measured by starting from the position where the maximum preperitoneal fat thickness is seen, to 20 mm in the caudal direction (Figure 1B). Subcutaneous fat thickness reflects subcutaneous central fat mass and preperitoneal fat is found to be a proxy of visceral abdominal fat.[19] Both preperitoneal and subcutaneous fat are associated with metabolic syndrome outcomes. The ratio between this preperitoneal fat thickness and subcutaneous thickness, based on ultrasound measures, is a useful method to estimate the abdominal fat distribution. A higher ratio reflects a more adverse abdominal fat distribution. This method has been used in several studies.[14] We performed a validation study among 34 children (aged 1-18 years) and showed that abdominal fat measurements by ultrasound were strongly correlated with measurements obtained by Computer Tomography (CT). Of the total group 9, children were between the ages of 1 and 4 years. Overall correlation coefficients ranged from 0.75 to 0.97 for the whole group and from 0.56 to 0.94 for children younger than 4 years. To assess the intra-observer repeatability of our measurements we calculated intra-class correlation coefficients (ICC's). For the ultrasound measurements the ICC's ranged from 0.973 to 0.976 from which we can conclude that our measurements for ultrasound were highly reproducible. Results were similar for children younger and older than 4 years. Agreement between ultrasound and CT measurements was assessed using Bland and Altman plots and showed small systematic differences between the measurements obtained by ultrasound and CT. Thus, measuring abdominal fat distribution using ultrasound is a useful method for epidemiological research in children, but should be used carefully for obtaining absolute measurements in individual children.

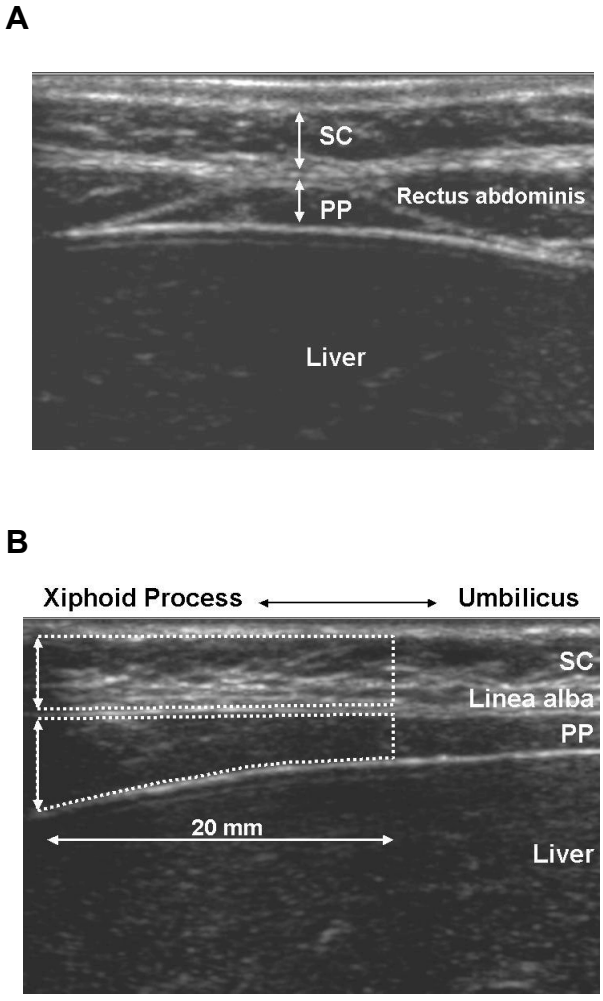
Anthropometrics of the child

Weight was measured at the age of 2 years by a mechanical personal scale (SECA). Height was measured by a Harpenden stadiometer (Holtain Limited) in standing position. Body mass index (kg/m^2) and body surface area (m^2) were calculated.

Covariates

Information on maternal age and weight was obtained by the first questionnaire at enrollment in the study. Maternal weight gain during pregnancy was calculated as

Figure 1: Measurements of abdominal fat with ultrasound.



*Transversal images from an ultrasound (**A**) at the location where the subcutaneous (SC) thickness was measured. Sagittal images from an ultrasound (**B**) where the maximum preperitoneal (PP) thickness and the PP and SC area were measured starting at the location of the maximum PP thickness to 20 mm in caudal direction.*

pre-pregnancy to third trimester weight change. Since enrolment in our study was in early pregnancy, we were not able to measure maternal weight before pregnancy. We obtained information about pre-pregnancy weight by questionnaire. Correlation of pre-pregnancy weight obtained by questionnaire and weight measured at enrolment was 0.97 ($P < 0.001$). Maternal height was measured without shoes at our research center and pre-pregnancy body mass index (kg/m^2) was calculated. In total, 4 of the 481 mothers did have gestational diabetes and 45 of the 481 subjects did have a family history

of diabetes. This information was obtained from midwife and hospital registries and questionnaires, respectively. Information on duration of breastfeeding was collected by questionnaires at 2, 6 and 12 months.

Statistical analysis

Differences between boys and girls were examined with Student's t-tests and Chi square tests. The associations of fetal weight in the second and third trimester of pregnancy and birth weight with abdominal fat mass were assessed using linear regression models. For this purpose we calculated relative fat mass (%) at the age of 2 years as percentage of abdominal fat mass. The interpretation of the differences in abdominal fat mass is easier by using relative differences expressed as percentages. To compare the effects of weight at different ages, the effects are presented per change in standard deviation (SD) score. Since preperitoneal area and the preperitoneal/subcutaneous ratio were not normally distributed, log transformation was applied and effect estimates are presented as geometric means. These regression models were adjusted for age at visit (months), sex, breastfeeding and current body mass index. Since, no associations were found for maternal pre-pregnancy weight and pregnancy weight gain, maternal age, smoking and paternal anthropometrics with abdominal fat mass measures and adding these variables to the regression models did not materially change the effect estimates, they were not included in the final models. Similarly, we additionally adjusted our regression models for gestational diabetes, family history of diabetes and birth weight using a multiple regression analysis. Since our effect estimates did not change, these covariates were not included in the final models. Similar regression models were used to assess the effect of fetal and postnatal weight change (SD) on abdominal fat mass measures. Next, we constructed tertiles of birth weight and used linear regression models to examine the effect of catch-down and catch-up growth on abdominal fat mass compared to non-changers. These models were adjusted for age at visit (months), sex and breastfeeding. Tests for trends within strata were performed by using the continuous variables in the fully adjusted linear regression model. To test whether the associations of birth weight and postnatal growth tertiles with abdominal fat mass measures were modified by sex, we added interaction terms of sex with birth weight and postnatal growth to our regression models. These were not significant (all p-value > 0.1) and therefore not further used in the models. We calculated that with $\alpha = 0.05$ and 80% power, we were able to detect differences of 0.13 SD (SD 0.2 for both independent and dependent variables). We are not aware of previous studies that studied the associations of growth characteristics in early life with abdominal fat mass measures in childhood. However, previous studies focused on body mass index as outcomes showed much larger effect estimates.[20] All measures of association are presented with their 95% confidence intervals (CI's). Statisti-

cal analyses were performed using the Statistical Package of Social Sciences version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Table 1 presents the clinical characteristics of the children and their mothers. Of all participating children, 51% were male. Birth weight was higher in boys than in girls. No differences were found between boys and girls in breastfeeding. At the age of 2 years, girls had a higher subcutaneous transversal distance and area (differences 10.27 % (95% CI 3.82, 16.72) and 11.13 % (95% CI 4.49, 17.78), respectively. Table 2 shows that weight, body surface area and body mass index were all strongly positively associated with all abdominal fat measures. The smallest effect estimates were found for height.

Table 3 shows weak tendencies towards positive associations of fetal weight with both subcutaneous fat distance and area. These were all not significant. Tendencies towards inverse associations of fetal weight with preperitoneal fat were found. The strongest effect was found for second trimester fetal weight, which was inversely associated with preperitoneal fat area (-3.73 (95% confidence interval -7.23, -0.10) % per standard deviation score (SDS) increase in weight. Fetal weight in second and third trimester were inversely associated with the preperitoneal/subcutaneous ratio. The associations were of borderline significance. No significant associations between birth weight and abdominal fat measures were found. Weight gain from birth to the age of 2 years was positively associated with preperitoneal fat mass measures ($p < 0.01$). No associations were found between postnatal weight gain and subcutaneous fat mass measures.

Table 4 shows that within each tertile of birth weight catch up growth was positively associated with subcutaneous and preperitoneal fat mass measures. Tests for trend for the ratio preperitoneal/subcutaneous distance were not significant. Except for subcutaneous area, the lowest and highest fat mass percentages, were found in children in the lowest birth weight tertile with catch down growth and in children in the highest birth weight tertile with catch up growth, respectively. Differences up to 22% were observed.

Discussion

This population-based prospective cohort study showed that second trimester estimated fetal weight were not associated with abdominal subcutaneous fat mass measures but showed tendencies towards inverse associations with preperitoneal fat measures at the age of 2 years, which are related to abdominal visceral fat mass. Birth weight was not associated with abdominal fat mass measures. Weight gain from birth to the age of

Table 1: Maternal, fetal and child characteristics (n = 481).

Maternal characteristics	
Age (years)	31.9 (3.8)
Weight (kg)	69.0 (13.3)
Height (cm)	170.9 (6.2)
Body Mass Index (kg/m^2)	23.6 (4.3)
Smoking habits (%)	
Never smoked	83%
Smoked until pregnancy was known	7%
Throughout pregnancy	10%
Fetal and child characteristics	
Second trimester	
Gestational age (weeks)	20.5 (19.0 – 22.0)
Estimated fetal weight (grams)	366 (69)
Third trimester	
Gestational age (weeks)	30.3 (28.7 – 32.2)
Estimated fetal weight (grams)	1,614 (254)
Birth	
Gestational age (weeks)	40.1 (37.4 – 42.1)
Weight (grams)	3,501 (529)
Low birth weight (<2500 grams) %	3.5
Preterm birth (%)	3.3
Males (%)	51
2 years	
Age at visit (months)	25.3 (23.4- 27.9)
Weight (grams)	12,550 (1,358)
Height (cm)	88.9 (3.3)
Body Surface Area (m^2)	0.56 (0.04)
Body Mass Index (kg/m^2)	15.8 (1.3)
Breastfeeding	
Ever (%)	91
Duration (months)	4.9 (0.5 – 12.0)
Abdominal measures at 2 years	
Subcutaneous transversal distance (mm)	2.88 (1.37 – 4.80)
Subcutaneous area (mm^2)	45.24 (23.0 – 79.0)
Preperitoneal distance (mm)	2.26 (1.33 – 3.47)
Preperitoneal area (mm^2)	28.76 (17.0 – 19.45)
Ratio preperitoneal/ subcutaneous transversal distance	1.07 (0.58 – 1.79)

Values are means (SDS), percentages or medians (90% range). Of the total group, data were missing on weight before pregnancy (n = 61), maternal height at intake (n = 3), BMI before pregnancy (n = 62), gestational age second trimester (n = 15), estimated fetal weight second trimester (n = 20), gestational age third trimester (n = 15), estimated fetal weight third trimester (n = 21), age at visit at 2 years (n = 7), current weight (n = 9), current height (n = 22), BMI at 2 years (n = 22), ever breastfeeding (n = 4), duration of breastfeeding (n = 57), SC trans (n = 3), SC area (n = 12), PP distance (n = 8), PP area (n = 11) and ratio PP/SC (n = 8).

Table 2: Associations between anthropometrics and abdominal fat measures (%) at the age of 2 years.

	Abdominal fat measures (%)				
	Subcutaneous transversal distance	**Subcutaneous area**	**Preperitoneal distance**	**Preperitoneal area**	**Ratio preperitoneal/ subcutaneous distance**
Height (1 SD = 3.3 cm)	1.94 (-1.39, 5.31) P = 0.25	1.49 (-2.00, 4.98) P = 0.40	3.19 (0.35, 6.03) P = 0.03	4.60 (1.51, 7.79) P < 0.01	1.11 (-2.18, 4.50) P = 0.51
Weight (1 SD = 1.4 kg)	12.49 (9.37, 15.65) P < 0.01	11.88 (8.63, 15.13) P < 0.01	6.34 (3.59, 9.09) P < 0.01	8.33 (5.23, 11.52) P < 0.01	-5.54 (-8.52, -2.57) P < 0.01
BSA (1 SD = 0.04 m^2)	10.44 (7.18, 13.67) P < 0.01	9.88 (6.52, 13.25) P < 0.01	5.85 (3.06, 8.64) P < 0.01	7.68 (4.60, 10.96) P < 0.01	-4.30 (-7.41, -1.19) P < 0.01
BMI (1 SD = 1.3 kg/m^2)	15.30 (12.21, 18.39) P < 0.01	14.75 (11.56, 17.94) P < 0.01	5.63 (2.84, 8.42) P < 0.01	6.82 (3.77, 9.97) P < 0.01	-8.61 (-11.40, -5.64) P < 0.01

Values are regression coefficients (95% confidence interval) and reflect the difference (%) in abdominal fat mass measures for each SD change. Preperitoneal area and ratio values are geometric means. BSA = body surface area; BMI = body mass index.

Table 3: Associations of fetal and postnatal weight and weight gain with abdominal fat mass measures (%) at the age of 2 years.

	Abdominal fat measures (%)				
	Subcutaneous transversal distance	**Subcutaneous area**	**Preperitoneal distance**	**Preperitoneal area**	**Ratio preperitoneal/ subcutaneous distance**
Weight (SDS)					
Second trimester	1.46 (-2.50, 5.38) P =0.47	0.68 (-3.43, 4.79) P = 0.75	-3.24 (-6.69, 0.22) P = 0.07	-3.73 (-7.23, -0.10) P = 0.05	-3.34 (-7.13, 0.50) P =0.09
Third trimester	1.46 (-2.08, 5.00) P =0.42	1.49 (-2.24, 5.21) P = 0.43	-1.99 (-5.14, 1.15) P = 0.21	-0.60 (-4.02, 2.84) P = 0.72	-3.54 (-6.95, 0) P = 0.05
Birth	0.28 (-3.23, 3.75) P = 0.88	-1.05 (-4.70, 2.60) P = 0.57	-1.91 (-5.01, 1.20) P = 0.23	-1.00 (-4.21, 2.43) P = 0.58	-0.70 (-4.21, 2.84) P = 0.68
Weight change (SDS)					
3rd trimester - birth	-1.01 (-4.58, 2.57) P = 0.58	-2.59 (-6.35, 1.16) P = 0.18	0.09 (-3.10, 3.28) P = 0.95	-0.30 (-3.63, 3.25) P =0.88	2.84 (-0.80, 6.61) P = 0.13
Birth – 2 years	1.08 (-2.50, 4.61) P =0.56	2.13 (-1.59, 5.84) P = 0.26	3.50 (0.31, 6.65) P =0.03	3.77 (0.30, 7.36) P =0.04	0.60 (-2.96, 4.29) P = 0.74

Values are regression coefficients (95% confidence interval) and reflect the difference (%) in abdominal fat mass measures for change in SD in weight. Models are adjusted for age at visit (months), sex, breastfeeding and current body mass index. Preperitoneal area and ratio values are geometric means.

Table 4: The associations between catch down and catch up growth with abdominal fat mass (%).

	Postnatal growth			
	Catch down growth (n = 163)	Non-changers (n = 215)	Catch up growth (n = 76)	
Subcutaneous transversal distance				
Birth weight				
1st tertile (n = 160)	-17.14 (-34.42, 0.14)	-3.96 (-16.07, 8.12)	5.27 (-7.95, 18.49)	P trend = 0.02
2nd tertile (n = 152)	-15.16 (-29.01, -1.32)	**Reference**	18.56 (1.63, 35.50)	P trend < 0.01
3rd tertile (n = 157)	-4.86 (-16.07, 6.32)	11.73 (-0.80, 24.29)	-15.27 (-65.68, 35.15)	P trend = 0.02
	P trend < 0.01	P trend < 0.01	P trend = 0.08	
Subcutaneous area				
Birth weight				
1st tertile (n = 160)	-17.96 (-35.28, -0.63)	-7.44 (-19.78, 4.90)	6.01 (-7.36, 19.38)	P trend < 0.01
2nd tertile (n = 152)	-10.81 (-24.82, 3.19)	**Reference**	16.13 (-0.82, 33.07)	P trend < 0.01
3rd tertile (n = 157)	-10.91 (-22.21, 0.39)	14.29 (1.63, 26.94)	2.25 (-48.12, 52.63)	P trend < 0.01
	P trend < 0.01	P trend < 0.01	P trend = 0.07	
Preperitoneal distance				
Birth weight				
1st tertile (n = 160)	-21.99 (-33.73, -7.76)	-8.55 (-18.71, 1.55)	-3.24 (-14.14, 7.62)	P trend = 0.04
2nd tertile (n = 152)	-16.45 (-27.93, -4.96)	**Reference**	2.62 (-11.30, 16.49)	P trend = 0.02
3rd tertile (n = 157)	-11.04 (-20.30, -1.82)	-2.53 (-12.90, 7.85)	14.45 (-19.46, 48.36)	P trend = 0.07
	P trend = 0.02	P trend = 0.33	P trend = 0.95	
Preperitoneal area				
Birth weight				
1st tertile (n = 160)	-24.87 (-35.40, -12.54)	-6.95 (-16.47, 3.67)	1.21 (-9.97, 13.77)	P trend < 0.01
2nd tertile (n = 152)	-15.38 (-25.10, -4.30)	**Reference**	5.76 (-8.88, 22.63)	P trend < 0.01
3rd tertile (n = 157)	-7.41 (-16.14, 2.22)	3.15 (-7.69, 15.26)	16.77 (-18.70, 67.70)	P trend = 0.10
	P trend < 0.01	P trend = 0.12	P trend = 0.80	
Ratio preperitoneal/subcutaneous distance				
Birth weight				
1st tertile (n = 160)	-1.09 (-16.31, 17.00)	0.30 (-10.95, 13.09)	-4.40 (-15.89, 8.76)	P trend = 0.34
2nd tertile (n = 152)	-3.54 (-15.72, 10.52)	**Reference**	-12.10 (-25.40, 3.56)	P trend = 0.28
3rd tertile (n = 157)	2.94 (-7.69, 14.80)	-11.57 (-21.81, -0.10)	-4.78 (-36.17, 42.05)	P trend = 0.05
	P trend = 0.28	P trend < 0.01	P trend = 0.02	

Values are regression coefficients (95% CI) and reflect the difference in abdominal fat measures (%) compared to children in the 2nd tertile of birth weight and without growth realignment. All model adjusted for age at visit (months), sex and breastfeeding. Preperitoneal area and ratio values are geometric means. Tests for trends within each stratum of birth weight and catch up growth were performed by using the continuous variables in the fully adjusted linear regression model.

2 years was positively associated with the preperitoneal abdominal fat mass measures, but not with subcutaneous abdominal fat mass measures. Similarly, we found tendencies towards positive associations between postnatal catch up growth and subcutaneous and preperitoneal fat mass measures in each tertile of birth weight.

To our knowledge, this is the first prospective cohort study examining the associations of fetal and postnatal growth characteristics with abdominal fat mass measured by ultrasound. Thus far, most studies focused on adiposity in early childhood used body mass index or waist-to-hip ratio as outcome.[9,21] However, abdominal visceral fat has been suggested to be stronger related to adverse metabolic syndrome outcomes and is therefore of greater interest. In this study, abdominal fat mass was measured by ultrasound, a valid method for measuring abdominal fat distribution in children in which preperitoneal fat mass is related to abdominal visceral fat mass.[14] The strength of this study is the population-based cohort with a relative large number of subjects studied with ultrasound. The study group consisted of healthy children participating in an ongoing prospective cohort study. Potential limitations of this study are the small numbers of preterm births and children born with low birth weight. Preterm born infants may be at risk of development of metabolic syndrome outcomes in later life through increased and aberrant adiposity.[22,23] These small numbers make it difficult to study the effect of preterm birth and low birth weight on abdominal fat mass and limits extrapolation of our results to this specific group of children. However, our results suggest similar effects of early growth characteristics on abdominal fat mass development in healthy children.

Previous studies have shown that accelerated postnatal growth is related to development of obesity and type 2 diabetes. Stettler et al. showed in the National Collaborative Perinatal Project among 19,397 participants that a rapid weight gain during the first 4 months of life leads to an increased risk of childhood overweight at the age of 7 years.[24] A similar association of weight gain in infancy with obesity in adulthood was found in a cohort of 300 African Americans. Rapid weight gain seems not only to be associated with obesity but also with metabolic syndrome outcomes. In a prospective birth cohort study among 851 children associations were found between early postnatal weight gain and decreased insulin sensitivity at the age of 8 years.[25] Recently, it was shown that postnatal catch up growth in the first 6 weeks of life led to higher total fat mass measured as skin fold thickness.[26] Highest growth rates in infancy seem to occur particularly in children born with low birth weight.[26] It is known that a majority of children born with low birth weight have a postnatal catch up growth during the first two postnatal years.[27] These findings suggest that especially children with low birth weight and high growth rates in infancy are at increased risk for developing metabolic syndrome outcomes. Our results are in line with these findings. Fetal and birth weight were inversely associated with preperitoneal fat mass. The largest effect was found for fetal weight in second trimester of pregnancy. Also, growth from birth to the age of 2 years was associated

with increased abdominal fat mass. Our results suggest that growth patterns that have previously been related to increased body mass index and development of metabolic syndrome outcomes in adults are also associated with an increased abdominal fat mass in early childhood. Catch up growth during the first two years was associated with increased levels of both subcutaneous and preperitoneal fat measures. However, the increase in subcutaneous fat mass measures was larger, leading to lower preperitoneal and subcutaneous fat area ratios. Thus, although catch up growth leads to an increase in abdominal adiposity, the distribution is not adversely affected.

Postnatal catch up growth is associated with both increased body mass index and abdominal mass. Body mass index could be an intermediate in the associations between postnatal catch up growth and abdominal fat mass in childhood. The models in table 4 were therefore not additionally adjusted for body mass index. Since previous studies have demonstrated adverse effects of increased abdominal fat mass in children and adults with the same body mass index, further studies in larger cohorts are needed to assess development of body mass index and abdominal adiposity, separately. To date no studies have assessed whether abdominal fat mass tracks from early childhood into adulthood. However, tracking of body mass index has been extensively studied and described.[28-30] The overall tracking coefficient from childhood to adulthood is about 0.6, but is strongly dependent on age and age interval.[31,32] Studies focused on tracking of abdominal fat mass from childhood to adulthood are important, since they may reveal insight in the early origins of obesity and metabolic syndrome. Consequences of abdominal fat mass in young children for metabolic readouts have not been studied yet. Studies in adults showed that abdominal visceral obesity leads to cardiovascular diseases and type 2 diabetes.[33] Additionally, it has been suggested that abdominal adiposity is associated with an increased risk of mortality.[9]

Several studies have shown positive associations between maternal and offspring anthropometrics, like height, weight and body mass index.[34,35] We did not find significant associations of maternal and paternal anthropometrics with abdominal fat mass measures in the offspring. However, no measures of abdominal adiposity or waist-hip circumference were available in parents. Further studies are needed to assess whether measures of abdominal fat mass in parents are related to similar measures in the offspring.

In conclusion, this study suggest that growth characteristics and patterns in late fetal and in early postnatal life are associated with increased abdominal fat mass in early childhood. Catch up growth after birth, even in the normal range of birth weight, is associated with higher abdominal fat mass accumulation. Abdominal fat measured by ultrasound may be an interesting biomarker for predicting later adverse outcomes. Although high abdominal fat mass is an important risk factor for cardiovascular disease, metabolic syndrome and mortality in adulthood, not much is known about the conse-

quences of increased abdominal fat mass in childhood. Further studies are needed to examine whether the associations persist in later life and are associated with development of metabolic syndrome outcomes.

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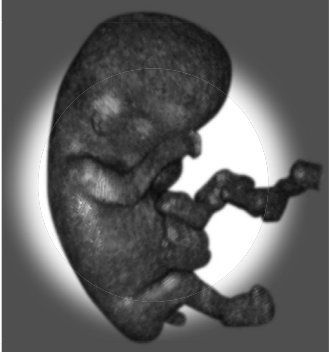

CHAPTER 2.5

Fetal and postnatal growth rates and the risk of obesity during early childhood

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Abstract

Background: We examined whether infant growth rates are influenced by fetal growth characteristics and are associated with the risks of overweight and obesity in early childhood.

Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards. Fetal growth characteristics (femur length and estimated fetal weight) were assessed in second and third trimester and at birth (length and weight). Infant peak weight velocity (PWV), peak height velocity (PHV) and body mass index at adiposity peak (BMIAP) were derived for 6,267 infants with multiple height and weight measurements. Definition of overweight and obesity at age 4 years were based on international standards.

Results: Estimated fetal weight in second trimester was positively associated with PWV and BMIAP during infancy (p-values for linear trends <0.05). Subjects with a smaller weight increase between 3rd trimester and birth had a higher PWV (p-value for linear trend <0.05). Second trimester femur length was positively associated with PHV (p-value for linear trend <0.05). Slower length gain during second and third trimester was associated with higher PHV after birth (p-values for linear trends <0.05). As compared to infants in the lowest quintile, those in the highest quintile of PWV and BMIAP had a strongly increased risks of overweight/obesity at the age of 4 years (Odds Ratios (95% CI): 15.01 (9.63, 23.38), and 47.28 (24.26, 92.12), respectively).

Conclusion: Fetal growth characteristics strongly influence infant growth rates. Infant growth patterns were also associated with the risk of overweight and obesity at 4 years of age. Longer follow-up studies are necessary to determine how fetal and infant growth patterns affect the risk of disease in later life.

Introduction

The inverse relationship between birth weight and adverse metabolic phenotypes in adulthood has been well established.[1-3] Increasing evidence suggest that also infant growth patterns such as rapid postnatal weight gain are risk factors for disease in later life.[4,5] Recent data from the Northern Finnish Birth Cohort 1966 Study suggest that infant growth characteristics such as the peak weight velocity (PWV) and peak height velocity (PHV) are predictors of increased blood-pressure, waist circumference and body mass index (BMI) at the age of 31 years.[6] Also, BMI at the adiposity peak (BMIAP), which occurs at around 9 months of age, was positively associated with BMI at the age of 31 years.[7] Growth rate in early postnatal life is highly dependent of birth weight, since smaller babies tend to catch-up and heavier babies tend to catch-down during the first months of postnatal life.[8] Birth weight is a crude measure of fetal growth as different fetal growth patterns may lead to the same birth weight.[9] Growth restriction during different critical periods of fetal growth can have different metabolic consequences in adult life.[10] An adverse environment has been demonstrated to influence fetal growth as early as the 10th week of pregnancy.[11] Infant growth rates and patterns might be intermediates in the association between impaired fetal growth and the increased risk of obesity and metabolic disease in later life. However, the associations between fetal growth characteristics and early postnatal growth rates are not known.

Therefore, we examined in a prenatally recruited prospective cohort study among 6,267 children whether infant growth rates are influenced by fetal growth characteristics and are associated with the risks of overweight and obesity in early childhood.

Methods

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study of 9,897 children and their parents from early fetal life onwards. This study is designed to identify early determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[12] Pregnant women were asked to enrol between 2001 and 2005, and enrolment was aimed for in first trimester but was allowed until birth. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. All participants gave written informed consent.

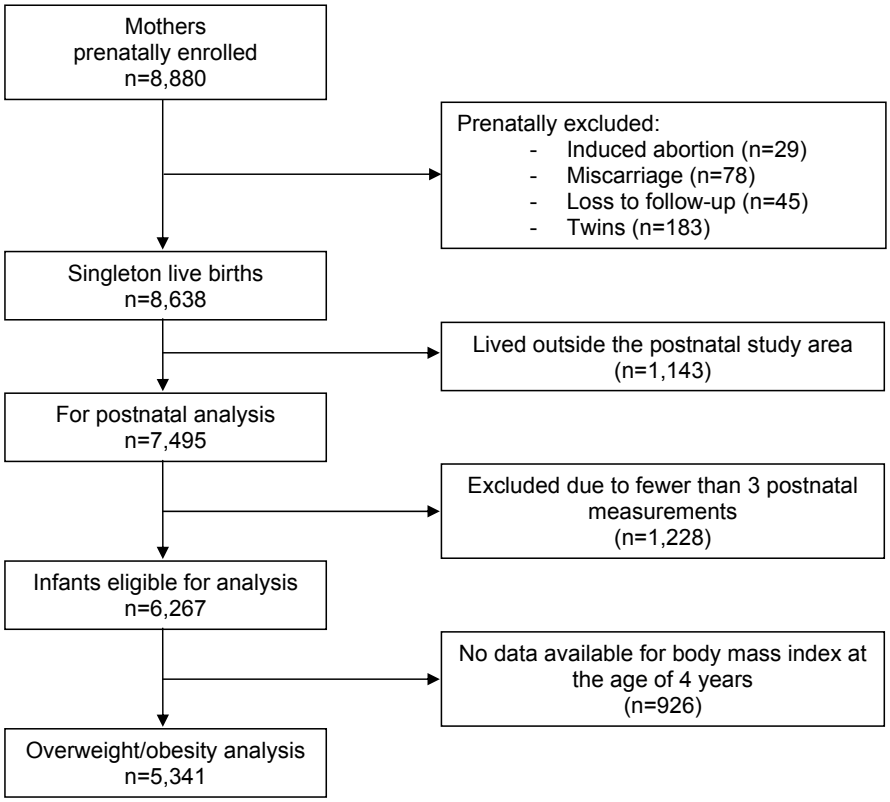
Population for analysis

In total, 8,880 mothers were recruited during pregnancy, and gave birth to 8,638 singleton live births (Figure 1). Of these children, 13 percent (n=1,143) lived outside the study area for postnatal follow-up, and 14 percent (n=1,228) children had fewer than three postnatal measurements, which is necessary for the infant growth modelling, leaving n=6,267 subjects for the analyses. Of these children, 85 percent (n=5,341) were available for the analyses regarding overweight and obesity at the age of 4 years.

Fetal growth measurements and birth outcomes

In a dedicated research facility, we measured fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) to the nearest millimeter using standardized ultrasound procedures in second and third trimester.[13] Estimated fetal weight (EFW) was calculated using the formula by Hadlock ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC)

Figure 1: Population for analysis.



+ 0.171 (FL) + 0.00034 (HC)^2 – 0.003685 (AC * FL)).[14] Standard deviation scores for all fetal growth characteristics were constructed on data from the study group.[13] Ultrasound examinations were performed using an Aloka® model SSD-1700 (Tokyo, Japan) or the ATL-Philips® Model HDI 5000 (Seattle, WA, USA). Fetal growth measurements were available in 6,004 and 6,181 children in second and third trimester, respectively. Fetal measurements in first trimester were not included as growth characteristics because these ultrasound examinations were primarily performed to establish gestational age. Date of birth, birth weight and length and infant sex were obtained from community midwife and hospital registries. Birth length was only available in 4,164 individuals (66.4%), since birth length is not routinely measured in obstetric practices in the Netherlands. Gestational age adjusted standard deviation scores (SDS) for birth weight and length, were constructed using growth standards from Niklasson.[15]

Postnatal growth measurements and derived infant growth parameters

Well-trained staff in the community health centers obtained postnatal growth characteristics (weight and length) using standardized procedures and BMI (kg/m^2) was calculated.[12] The median number of postnatal growth measurements was 5 (90% range: 3 – 8). Overweight and obesity were defined as described by Cole et al.[16]

Peak weight velocity and peak height velocity

PWV and PHV were derived from the postnatal data using the Reed1 model for boys and girls separately using the previously described procedure.[6,17] The Reed1 model[18] was chosen since it showed a better fit to the early growth data than the Kouchi, Carlberg and Count models, and it showed an equally good fit to the Reed2 model which has one more parameter than the Reed1 model. The Reed1 model was fitted by sex on all weight and height measurements taken at 0-4 years of age, including birth weight and length. We assumed both a fixed and random component for all four parameters in the model. For each person, the first derivative of the fitted distance curve was taken to get the weight or height velocity curve. Subsequently, the maximum of this curve was taken to obtain the PWV or PHV in infancy. The Reed1 model is a 4-parameter extension of the 3-parameter Count model[19] and its functional form is:[18]

$Y = A + Bt + C\ln(t) + D/t$

Since this model is not defined at birth (t=0), it was modified for this study in the same way as in Simondon et al:[20]

$Y = A + Bt + C\ln(t+1) + D/(t+1)$, where

t = postnatal age

Y = weight or height reached at age t

and A, B, C and D the function parameters.

Of the function parameters, *A* is related to the baseline weight or height at birth, *B* to the linear component of the growth velocity, *C* to the decrease in the growth velocity over time, and *D* to the inflection point that allows growth velocity to peak after birth rather than exactly at birth. The Reed1 model is linear in its constants.[19] Having two measurements was inadequate to capture the shape of the growth curve and therefore we restricted all association analyses to those with a minimum of three measurements per person.

Adiposity peak

For BMIAP, a cubic mixed effects model was fitted on log(BMI) from 14 days to 1.5 years, using sex as a covariate.[6] When fitting the model, age was centralized to 0.75 years. In addition to fixed effects, we included random effects for the constant and the slope in the model. We assumed autoregressive AR(1) within-person correlation structure between the measurements. Then, BMI at the point where the curve reaches its maximum, i.e. at infant adiposity peak, was derived for each individual.

Covariates

At enrolment data regarding maternal age, pre-pregnancy weight, parity and smoking was obtained by questionnaires.[12] Both parents were asked to provide details regarding the country of birth of their parents. This information was used to classify ethnic background of the child according to Statistics Netherlands, as previously described in detail.[21] Maternal height was measured at our research center and body mass index (BMI) was calculated (weight (kg) / height $(m)^2$). We obtained information regarding breastfeeding duration by postnatal questionnaires at the ages of 2, 6 and 12 months. Mothers were asked whether they ever breastfed their child and, if so, at what age the stopped breastfeeding.

Statistical analysis

First, using multivariate linear regression models and adjusting for covariates, we assessed the associations of estimated fetal weight in second and third trimester and birth weight with infant PWV and BMIAP. The covariates in the model were fetal ethnicity, maternal age, maternal educational level, maternal pre-pregnancy BMI, maternal smoking, parity, duration of breastfeeding, and number of postnatal measurements. Using similar models, we then examined whether weight change (in SDS) between second trimester and third trimester (second trimester weight gain), and between third trimester and birth (third trimester weight gain), were associated with infant PWV and BMIAP.

Subsequently, similar analyses were repeated for the associations of (femur) length with PHV and BMIAP. Since fetal body length can not be measured, femur length in second and third trimester was used as a proxy for body length.[22] Finally, using multivariate logistic regression models we assessed whether PWV, PHV and BMIAP were associated with the risk of overweight and obesity during infancy at the age of 4 years.[16] For this, PWV, PHV and BMIAP were stratified into quintiles and the lowest quintile was used as the reference category. Analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA) and R version 2.10.1 (The R Foundation for Statistical Computing).

Results

Subject characteristics are shown in Table 1. Of all children, 67% were of Caucasian ethnicity. The mean maternal age was 30.3 years, the median maternal weight was 67.0 kg, and the mean height was 167.7 cm.

Estimated fetal weight in second trimester was positively associated with PWV (p-value for linear trend <0.05) and BMIAP (p-value for linear trend <0.01) (Table 2). Also, we found a positive association between birth weight and BMIAP (p-value for linear trend <0.0001), while the association between birth weight and PWV was inverse (p-value for linear trend <0.001). Second and third trimester weight gain were both positively associated with BMIAP (both p-values for linear trends <0.0001). Infants with a smaller weight gain during third trimester had a higher PWV (p-value for linear trend <0.0001).

Second trimester femur length was positively associated with PHV (p-value for linear trend <0.05) and negatively associated with BMIAP (p-value for linear trend <0.01) (Table 3). At birth, these associations were both reversed where length was negatively associated with PHV and positively associated with BMIAP (p-values for linear trends <0.0001). Slower length gain during second and third trimester was associated with higher PHV after birth (p-values for linear trends <0.05). Third trimester length gain was positively associated with BMIAP (p-value for linear trend <0.0001)

Table 4 shows the associations between PWV, PHV and BMIAP with the risk of overweight and obesity at the age of 4 years. Subjects in the highest quintile of PWV had an increased risk of being overweight/obese obese at the age of 4 years (OR (95% CI): 15.01 (9.63, 23.38)). There was no association between PHV and the risk of overweight or obesity at 4 years. Compared to children in the lowest quintile of BMIAP, subjects in the highest quintile of BMI at adiposity peak had a strongly increased risk of overweight/obesity at the age of 4 years (OR (95% CI): 47.28 (24.26, 92.12)).

Table 1: Maternal and child characteristics (n=6,267)

Maternal characteristics	
Age (years)	30.3 (5.1)
Weight (kg)	67.0 (52.0 – 94.0)
Height (cm)	167.7 (7.4)
Body Mass Index (kg/m^2)	23.7 (19.4 – 33.3)
Educational level	
Primary (%)	9.2%
Secondary (%)	42.6%
Higher (%)	48.2%
Smoked during pregnancy (% yes)	23.9%
Parity (% primiparous)	56.3%
Fetal and child characteristics	
Sex (% males)	50.6%
Ethnicity	
Caucasian (%)	66.5%
Turkish (%)	7.6%
Surinamese (%)	7.0%
Moroccan (%)	6.1%
Other / mixed (%)	13.8%
Second trimester	
Gestational age (weeks)	20.5 (18.9 – 22.7)
Estimated fetal weight (grams)	380 (91)
Femur length (mm)	33.4 (3.5)
Third trimester	
Gestational age (weeks)	30.4 (28.9 – 32.2)
Estimated fetal weight (grams)	1,623 (254)
Femur length (mm)	57.4 (3.0)
Birth	
Gestational age (weeks)	40.1 (37.1 – 42.1)
Weight (grams)	3,442 (543)
Length (cm)	50.2 (2.4)
Infancy	
Number of postnatal measurements	5 (3 – 8)
Peak weight velocity (PWV) (kg/year)	12.3 (9.1 – 16.1)
Peak height velocity (PHV) (cm/year)	48.5 (38.7 – 64.9)
Adiposity peak, Body Mass Index (kg/m^2)	17.6 (0.8)
Breastfeeding duration (months)w	3.5 (0.5 – 12.0)

Values are means (standard deviation), percentages or medians (90% range)

Table 2: The association of (estimated fetal) weight with peak weight velocity and body mass index at adiposity peak.

Estimated fetal weight 2nd trimester (SDS)	**Peak weight velocity (PWV) (kg/year) (n=5,943)**	**Adiposity peak (BMI) (kg/m²) (n=5,421)**
1st quintile	12.02 (1.19)	17.52 (0.80)
2nd quintile	12.01 (1.18)	17.56 (0.80)
3rd quintile	12.12 (1.18)	17.60 (0.80)
4th quintile	12.22 (1.19)	17.64 (0.82)
5th quintile	12.16 (1.16)	17.68 (0.77)
p-value for linear trend	< 0.05	<0.01
Estimated fetal weight 3rd trimester (SDS)	**Peak weight velocity (PWV) (kg/year) (n=6,114)**	**Adiposity peak (BMI) (kg/m²) (n=5,598)**
1st quintile	12.00 (1.18)	17.41 (0.80)
2nd quintile	12.09 (1.19)	17.53 (0.80)
3rd quintile	12.18 (1.18)	17.64 (0.77)
4th quintile	12.12 (1.20)	17.67 (0.81)
5th quintile	12.16 (1.19)	17.80 (0.79)
p-value for linear trend	0.33	< 0.0001
Birth weight (SDS)	**Peak weight velocity (PWV) (kg/year) (n=6,265)**	**Adiposity peak (BMI) (kg/m²) (n=5,705)**
1st quintile	12.16 (1.18)	17.25 (0.78)
2nd quintile	12.23 (1.19)	17.46 (0.76)
3rd quintile	12.18 (1.19)	17.62 (0.76)
4th quintile	12.08 (1.19)	17.74 (0.76)
5th quintile	11.86 (1.20)	17.95 (0.79)
p-value for linear trend	< 0.001	< 0.0001
Weight change from 2nd to 3rd trimester (SDS)	**Peak weight velocity (PWV) (kg/year) (n=5,829)**	**Adiposity peak (BMI) (kg/m²) (n=5,332)**
1st quintile	12.16 (1.18)	17.50 (0.78)
2nd quintile	12.09 (1.19)	17.55 (0.79)
3rd quintile	12.08 (1.19)	17.59 (0.82)
4th quintile	12.11 (1.19)	17.63 (0.80)
5th quintile	12.05 (1.19)	17.75 (0.81)
p-value for linear trend	0.06	< 0.0001

Table 2: Continued

Weight change from 3rd trimester to birth (SDS)	Peak weight velocity (PWV) (kg/year) (n=6,141)	Adiposity peak (BMI) (kg/m²) (n=5,596)
1st quintile	12.39 (1.18)	17.43 (0.82)
2nd quintile	12.15 (1.19)	17.54 (0.78)
3rd quintile	12.14 (1.19)	17.64 (0.78)
4th quintile	12.09 (1.18)	17.71 (0.79)
5th quintile	11.78 (1.19)	17.78 (0.80)
p-value for linear trend	< 0.0001	< 0.0001

Values represent geometric means (standard deviation).
Model is adjusted for sex, age, fetal ethnicity, age of mother, maternal pre-pregnancy body mass index, maternal educational level, maternal smoking, parity, duration of breastfeeding and number of postnatal measurements.
SDS: Standard Deviation Score

Table 3: The association of (femur) length with peak height velocity and body mass index at adiposity peak.

Femur length 2nd trimester (SDS)	Peak height velocity (PHV) (cm/year) (n=5,802)	Adiposity peak (BMI) (kg/m²) (n=5,448)
1st quintile	48.89 (1.16)	17.67 (0.82)
2nd quintile	49.45 (1.18)	17.63 (0.76)
3rd quintile	48.73 (1.17)	17.62 (0.80)
4th quintile	49.48 (1.18)	17.55 (0.84)
5th quintile	49.28 (1.17)	17.54 (0.79)
p-value for linear trend	< 0.05	< 0.01
Femur length 3rd trimester (SDS)	**Peak height velocity (PHV) (cm/year) (n=5,993)**	**Adiposity peak (BMI) (kg/m²) (n=5,619)**
1st quintile	49.53 (1.18)	17.64 (0.82)
2nd quintile	49.21 (1.18)	17.66 (0.81)
3rd quintile	49.41 (1.17)	17.60 (0.79)
4th quintile	49.18 (1.17)	17.61 (0.79)
5th quintile	48.45 (1.16)	17.54 (0.80)
p-value for linear trend	0.64	< 0.01

Table 3: Continued

Birth length (SDS)	Peak height velocity (PHV) (cm/year) (n=4,125)	Adiposity peak (BMI) (kg/m²) (n=3,833)
1st quintile	56.26 (1.20)	17.46 (0.78)
2nd quintile	50.52 (1.16)	17.51 (0.80)
3rd quintile	48.51 (1.14)	17.62 (0.78)
4th quintile	46.76 (1.14)	17.66 (0.79)
5th quintile	43.22 (1.14)	17.77 (0.79)
p-value for linear trend	< 0.0001	< 0.0001
Length change from 2nd to 3rd trimester (SDS)	**Peak height velocity (PHV) (cm/year) (n=5,717)**	**Adiposity peak (BMI) (kg/m²) (n=5,369)**
1st quintile	49.82 (1.19)	17.57 (0.83)
2nd quintile	49.56 (1.17)	17.60 (0.79)
3rd quintile	49.01 (1.17)	17.61 (0.81)
4th quintile	48.71 (1.16)	17.62 (0.77)
5th quintile	48.53 (1.16)	17.62 (0.81)
p-value for linear trend	< 0.01	0.60
Length change from 3rd trimester to birth (SDS)	**Peak height velocity (PHV) (cm/year) (n=4,007)**	**Adiposity peak (BMI) (kg/m²) (n=3,789)**
1st quintile	55.47 (1.20)	17.42 (0.77)
2nd quintile	50.39 (1.16)	17.56 (0.79)
3rd quintile	48.40 (1.15)	17.56 (0.79)
4th quintile	47.04 (1.15)	17.70 (0.80)
5th quintile	44.10 (1.15)	17.81 (0.74)
p-value for linear trend	< 0.0001	< 0.0001

Values represent geometric means (standard deviation).
Model is adjusted for sex, age, fetal ethnicity, age of mother, maternal pre-pregnancy body mass index, maternal educational level, maternal smoking, parity, duration of breastfeeding and number of postnatal measurements.
SDS: Standard Deviation Score

Table 4: The association of peak weight velocity, peak height velocity and body mass index at adiposity peak with the risk of overweight/obesity[16] at the age of 4 years.

Peak weight velocity (PWV) (kg/year)	**Overweight/Obesity**
1st quintile	Reference
2nd quintile	2.70 (1.74, 4.19)***
3rd quintile	3.77 (2.43, 5.84)***
4th quintile	6.00 (3.88, 9.29)***
5th quintile	15.01 (9.63, 23.38)***
p for linear trend	< 0.0001
Peak height velocity (PHV) (cm/year)	**Overweight/Obesity**
1st quintile	Reference
2nd quintile	1.14 (0.83, 1.56)
3rd quintile	1.01 (0.73, 1.40)
4th quintile	0.82 (0.58, 1.16)
5th quintile	1.00 (0.70, 1.41)
p for linear trend	0.45
Body mass index at adiposity peak (kg/m²)	**Overweight/Obesity**
1st quintile	Reference
2nd quintile	3.46 (1.68, 7.14)***
3rd quintile	7.66 (3.86, 15.21)***
4th quintile	16.65 (8.54, 32.48)***
5th quintile	47.28 (24.26, 92.12)***
p for linear trend	< 0.0001

Overweight/obesity based on standard definitions established by Cole et al.[16]
Values represent odds ratio's (95% confidence interval) based on multivariate logistic regression.
Model is adjusted for sex, age, fetal ethnicity, age of mother, maternal pre-pregnancy body mass index, maternal educational level, maternal smoking, parity, duration of breastfeeding and number of postnatal measurements.
** p<0.05, ** p<0.01, *** p<0.001*

Discussion

We demonstrated strong associations between fetal growth characteristics and infant growth rates. The direction and size of the associations were dependent on the timing of the fetal growth variation. Second trimester estimated fetal weight was positively associated with both PWV and BMIAP during infancy. Slower third trimester weight and height gain, were associated with higher PWV and PHV, respectively. Both higher PWV and BMIAP during infancy were strongly positively associated with an increased risk of overweight and obesity at the age of 4 years.

To our knowledge, this is the first study that examined the associations of between infant growth rates with both fetal growth characteristics and the risk of overweight and

obesity in childhood. Analyses were performed in a large sample which made our study well-powered. Furthermore, data were available for a large number of covariates. A limitation might be that 16.4% of the children had fewer than three postnatal measurements and were therefore not included in the analyses. A minimum of three measurements was set for the postnatal growth modelling. Birth weight and birth length were lower in children without postnatal data available for analyses (70.6 (95% CI: 42.8, 98.4) grams and 0.26 (95% CI: 0.06, 0.46) cm, respectively). Also, birth length was missing in 33.6% of our sample, since this measurement is not a part of the routine obstetric practice in the Netherlands. Subjects without birth length measurement had a slightly smaller femur length in second and third trimester (p=0.07 and p=0.04, respectively) and a lower PHV (-0.60 (95% CI: -1.05, -0.16) cm/year). Smaller babies at birth are more likely to show lower growth rates in third trimester and increased growth rates during early infancy than normal size newborns. Therefore, we expect that this selection most likely will lead to an underestimation of inverse association between growth rates in third trimester and peak growth velocity during infancy.

Recently, it was demonstrated a population-based study from Finland that both PWV and PHV in the first months after life were associated with increased risk of higher blood pressure and BMI in adulthood.[6] Previously, catch-up growth or upward growth re-alignment in the first two years of postnatal life was shown to be associated with an adverse adult metabolic phenotype.[5,23] Moreover, it has been shown that children who were born small-for-gestational-age and had a rapid weight gain in the first 3 months of life were at increased risk of development of risk factors for cardiovascular disease and type 2 diabetes.[24]. It seems, that rapid weight gain in the first months immediately after birth may be of greater importance than catch up growth during the first two years.[25] Adaptations in early postnatal growth rates are influenced by a drive to compensate for prenatal fetal growth restriction or growth acceleration caused by the maternal-uterine environment.[26] In our study, we indeed found that there was a strong negative association between third trimester weight or height gain, and PWV and PHV during infancy. In contrast, growth in weight and height until second trimester was positively associated with PWV and PHV, respectively. Body stature and size are known to be a highly heritable trait, with a large genetic component.[27,28] It could be hypothesized that the fetus grows along its growth curve during the first half of pregnancy, but that this curve is more susceptible to maternal-uterine factors during late pregnancy. After birth, however, the child may continue along its original genetically determined growth curve, or may deviate from this due to compensatory accelerated or decelerated growth as a response to decreased or increased fetal third trimester growth respectively.

Obesity, measured by an increased BMI, has been suggested to track from childhood into adulthood.[29] We found that a higher BMIAP during infancy was associated with an increased risk of overweight/obesity at the age of 4 years. Similar odds ratios were found

when analyses were performed on the risk of obesity separately (data not shown), but due to the low number of obese subject (2.3%) this group was combined with overweight subjects. In the Northern Finnish Birth Cohort Study 1966, it was also found that BMI at adiposity peak was associated with higher BMI at 31 years of age.[7] In our study, estimated fetal weight at second trimester was positively associated with BMIAP. Also, birth weight itself was strongly positively associated with BMI at the age of 4 years (data not shown). Previously in the 1958 Birth Cohort, a J-shaped association between birth weight and BMI in adulthood was suggested.[30] Based on data from the current study, it could be hypothesized that fetuses that show third trimester growth restriction in late pregnancy, which might lead to a lower birth weight, show rapid weight gain postnatally and thus are at increased risk of developing obesity in later life. In contrast, fetuses that grow in the highest percentiles for weight, from second trimester onwards, are more likely to continue following this curve during postnatal life, which ultimately could lead to a higher BMI as adults.

In conclusion, we demonstrated strong associations between fetal growth characteristics and infant growth rates. Estimated fetal weight at second trimester was positively associated with a higher PWV during infancy. Both slower third trimester weight gain and height gain were strongly associated with higher postnatal PWV and PHV, respectively. Higher PWV and BMIAP are strong predictors of childhood overweight and obesity. Results from our study suggest that studies relating birth size with outcomes in later life should take the longitudinal fetal and infant growth measures into account. Longer follow-up studies are necessary to determine how infant growth patterns affect the risk of disease in later life.

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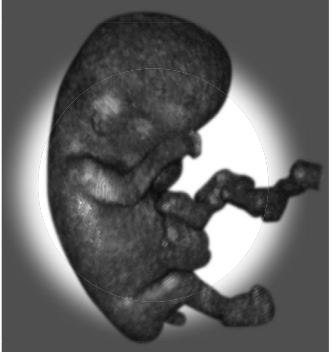
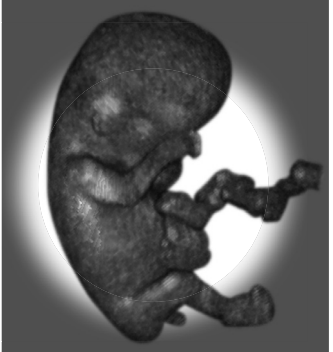


CHAPTER 3

Obesity and Type 2 diabetes genes and early growth



CHAPTER 3.1

Obesity gene *FTO* and body composition at the age of six months

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Abstract

Background: Genome-wide association studies on body mass index have identified an *FTO* polymorphism (rs9939609) as having the strongest effect. We examined the effect of *FTO* genotype on body composition at the age of 6 months using skinfold thickness measurements and Dual Energy X-ray Absorptiometry (DXA).

Methods: This study was embedded in a population-based prospective cohort study from early fetal life onwards. *FTO* genotype was related to anthropometric measurements (weight and height), subcutaneous fat mass measured by skinfold thickness, and total, truncal and peripheral fat mass and lean mass measured by DXA. Analyses for skinfold thickness and DXA were performed in 695 and 216 children, respectively.

Results: Genotype frequency was TT 40.3%, TA 45.5% and AA 14.2%. No significant differences between *FTO* genotypes were found in weight, height or body mass index. Furthermore, *FTO* genotype was not associated with any skinfold thickness. Finally, no associations between *FTO* genotype and body composition measures (fat and lean mass) assessed by DXA were found.

Conclusions: We observed no association between this *FTO* polymorphism and body composition at the age of 6 months. Longer follow-up studies are necessary to examine at which age and by which mechanisms *FTO* genotype starts to influence fat mass and body composition.

Introduction

Genome-wide association studies on body mass index have identified an *FTO* polymorphism (rs9939609) as having the strongest effect.[1] In a meta-analysis, each variant allele of this *FTO* polymorphism was shown to have an effect of up to 0.33 kg/m^2 in adulthood.[1] Since the initial identification of *FTO*, the association with adult obesity has been replicated in several populations.[2-5] Furthermore, other measures of body composition such as waist-hip ratio have also been shown to be affected by this *FTO* polymorphism.[6]

Recently, some studies have indicated that *FTO* genotype may already have an effect on body mass index in childhood.[7-12] The expression of *FTO* seems to become stronger throughout childhood, thus leading to an increasing effect on body mass index (BMI) over time.[12] Furthermore, the children with this variant have been shown to eat more and consume more fat and total energy.[7,13] The effect of *FTO* on early growth and development is important, since growth in early life is related to several health outcomes.[14] Though the exact pathway by which body composition is influenced by *FTO* remains unclear, studies in humans have indicated that the effect is most likely mediated through appetite and energy intake, rather than a metabolic mechanism, such as energy expenditure or lipid metabolism.[7,8,10,15,16] However, a recent functional study in Fto-deficient mice demonstrated that these mice had an increased expenditure, indicating that Fto may also be involved in energy homeostasis.[17]

Dual Energy X-ray Absorptiometry (DXA) is an easy to perform and precise technique to determine body composition in children.[18] In a large cohort in over 5,000 children aged 9 years, the risk allele of *FTO* gene was associated with an increased total fat mass measured by DXA.[11] Another smaller study in 234 newborns showed a similar increase in fat mass by means of DXA in the variant carriers of this *FTO* polymorphism as early as two weeks after birth.[9] No other data are available on the effect of this *FTO* polymorphism on fat mass measured by DXA during infancy.

Therefore, we examined the effect of *FTO* on body composition measured by DXA and subcutaneous fat mass measured by skinfold thickness at the age of six months in a prospective birth-cohort study.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. This study is designed to identify early environmental and genetic determinants of growth, development and health from

fetal life until young adulthood and has been described previously in detail.[19,20] Detailed measurements were performed using ultrasound and physical examinations, biological samples and advanced imaging techniques. This study was performed in a subgroup of children participating with additional detailed measurements. This subgroup is ethnically homogeneous to exclude possible confounding or effect modification by ethnicity. Dutch ethnicity was defined as having two parents and four grandparents born in the Netherlands. In children who were successfully genotyped, data on anthropometrics were available in 703 children. Of these subjects, skinfold thickness and DXA was available in 695 and 216 subjects, respectively. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Genotyping

DNA was collected from cord blood samples at birth. Cord blood for DNA isolation was available in 59% of all live born participating children. Missing cord blood samples were mainly due to logistical constraints at delivery. Genotyping of the *FTO* polymorphism (rs9939609) was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 minutes), then 40 cycles of 94° C (15 seconds) and 60° C (1 minute)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 96.2% of the samples. To confirm the accuracy of the genotyping results, 5% of the children were randomly selected and were genotyped for a second time with the same method. The error rate was less than 1%. Genotype frequency was TT 40.3%, TA 45.5% and AA 14.2% and the genotype distribution did not deviate from the Hardy-Weinberg equilibrium (p=0.57).

Birth characteristics, breastfeeding and anthropometric measurements

Birth weight, date of birth and sex were obtained from community midwife and hospital registries. Information about duration of breastfeeding was obtained by postnatal questionnaires at the ages of 2 and 6 months. At the age of 6 months subjects visited the research center. Anthropometrics were measured without clothes. Weight was measured to the nearest gram using electronic scales (SECA ®). Length was measured to the nearest millimeter in supine position using a neonatometer (Holtain Limited ®). Body mass index and ponderal index were calculated (weight / height^2 and weight / height^3, respectively).

Dual Energy X-ray Absorptiometry (DXA) measurements

Fat and lean mass were measured by Lunar Prodigy DXA scanner ® (General Electrics, Hoevelaken, the Netherlands) at the age of 6 months. Previous studies have shown that this method is valid for measurement of body composition in children and adolescents.[21] All DXA scans were performed by the same technician with the same scanner and software. Subjects were invited to participate in the DXA study during a limited period of the study. Children were not sedated during the scan. After the exclusion of scans with anomalies such as movement artifacts, complete scans were available for 216 infants with *FTO* genotype. The DXA scans were used to derive total, truncal, peripheral fat mass and lean mass. Details have been described previously.[22]

Subcutaneous fat mass

Skinfold thicknesses (SFT) were measured at the age of 6 months on the left side of the body at four different sites (biceps, triceps, suprailiacal and subscapular) according to standard procedures by using a skinfold caliper (Slim Guide, Creative Health Products®). Only subjects with measurements at all four sites were included for analyses. Four well-trained medical assistants performed all measurements.[23] Total subcutaneous fat mass was calculated from the sum of biceps SFT + triceps SFT + suprailliacal SFT + subscapular SFT. Central subcutaneous fat mass was calculated from the sum of suprailliacal SFT + subscapular SFT. Peripheral subcutaneous fat mass was calculated from the sum of triceps SFT + biceps SFT.

Data Analysis

First, we assessed the effect of *FTO* genotype on growth characteristics (weight, height, body mass index and ponderal index) using multivariate linear regression. Since not all children were measured exactly at the same age (median 6.3 months (90% range: 5.5, 7.8)), these models were adjusted for (1) age at visit (in months) and sex (coded as 1 for males, 2 for females). Subsequently this model was additionally adjusted for (2) birth weight (in grams), and (3) breastfeeding duration (in months), since these covariates are known to influence early growth. For these linear regression models, we assumed an additive genetic effect per A-allele (coded as 0, 1 or 2 A-alleles) based on previous studies.[11,13] Thus the three linear regression models can be written as follows:

Model 1: Growth characteristic = FTO genotype + age at visit + sex.
Model 2: Growth characteristic = FTO genotype + age at visit + sex + birth weight.

Model 3: Growth characteristic = FTO genotype + age at visit + sex + birth weight + breastfeeding duration.

Then, using these same three models, we examined the effect of genotype on subcutaneous fat mass measured by skinfold thickness, and body composition (fat mass, lean mass, total mass and fat percentage) measured by DXA. Statistical analyses were performed using the Statistical Package of Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics are shown in Table 1. DXA measurements were performed in 216 of the 703 children. There were no differences in birth weight and anthropometric measures at the age of 6 months between those children with and without DXA measurements (p=0.46 for birth weight, p=0.70 for weight 6 months and p=0.30 for height). After adjusting for sex and age, no differences were found for weight, height, body mass index and ponderal index between the three *FTO* genotypes (Table 2). Also in the adjusted models, no associations were found between genotype and these anthropometric measures. Table 3 shows subcutaneous fat mass measured by skinfold thickness (peripheral, central and total subcutaneous fat mass) per *FTO* genotype. No association was found between *FTO* genotype and any of the subcutaneous fat mass measurements, also not in the adjusted models. In Table 4, body composition measures (fat mass, lean mass, total tissue mass and fat percentage) assessed by DXA are shown by *FTO* genotype. No associations between *FTO* genotype and body composition measures assessed by DXA were found.

Discussion

We observed no association between this *FTO* gene polymorphism and body composition, assessed by anthropometrics measurements, skinfold thickness and DXA, in a healthy, ethnically homogenous population of 6 month old infants.

Only two previous studies have examined the effect of *FTO* genotype on body composition measured by DXA in children. In a study performed in 234 newborns, López-Bermejo *et al*. showed that at the age of 2 weeks children with the AA genotype already had increased total, truncal and abdominal fat mass.[9] Furthermore, they demonstrated that at birth, the A-allele was associated with increased concentrations of visfatin, an adipocytokine expressed in visceral fat. In this study, however, the association between

Table 1: Subjects characteristics of 703 children.

Birth characteristics	
Boys (%)	51.6%
Birth weight (grams)	3549 (487)
Gestational age (weeks)	40.2 (1.4)
Age 6 months	
Age at visit (months)	6.5 (0.7)
Weight (grams)	7933 (872)
Length (cm)	68.8 (2.6)
Body mass index (kg/m^2)	16.8 (1.3)
Ponderal index (kg/m^3)	24.4 (2.1)
Breastfeeding duration (months)	3.5 (0 – 6.5)
Skinfold thickness	
Peripheral fat mass (mm)	14.3 (3.8)
Central fat mass (mm)	12.6 (3.4)
Total fat mass (mm)	26.9 (6.2)
Fat Mass (DXA)	
Truncal (grams)	662 (201)
Peripheral (grams)	957 (277)
Total (grams)	1935 (487)
Lean Mass (DXA)	
Truncal (grams)	2969 (307)
Peripheral (grams)	1901 (297)
Total (grams)	6439 (641)
Total Mass (DXA)	
Truncal (grams)	3631 (407)
Peripheral (grams)	2858 (516)
Total (grams)	8374 (955)
Fat Percentage (DXA)	
Truncal (%)	18.0 (4.2)
Peripheral (%)	33.0 (5.0)
Total (%)	22.9 (4.0)
Truncal / Peripheral Ratio	0.54 (0.07)

Values represent percentages, means with standard deviation or median with 90% range.
Data at 6 months was missing on breastfeeding duration (n=41), skinfold thickness (n=8).
Fat percentage measured by DXA was available in 216 children.
DXA: Dual Energy X-ray Absorptiometry.

FTO genotype and over fifteen clinical characteristics were tested without adjusting for multiple hypotheses testing, which was acknowledged by the investigators. If one would have applied a Bonferroni correction, only the association of *FTO* with visfatin at birth

Table 2: Growth characteristics in 703 children by *FTO* genotype.

	TT (n=283)	TA (n=320)	AA(n=100)	p-value* Model 1	p-value* Model 2	p-value* Model 3
Age (months)	6.5 (0.7)	6.4 (0.7)	6.5 (0.8)	N/A	N/A	N/A
Weight (grams)	7958 (876)	7899 (876)	7971 (855)	ns	ns	ns
Height (cm)	68.7 (2.8)	68.7 (2.6)	69.0 (2.3)	ns	ns	ns
Body mass index (kg/m^2)	16.8 (1.3)	16.7 (1.3)	16.7 (1.3)	ns	ns	ns
Ponderal index (kg/m^3)	24.5 (2.2)	24.3 (2.1)	24.3 (2.0)	ns	ns	ns

Values represent means with standard deviation.
** p-values assume additive models.*
ns: not significant

Model 1: Adjusted for age at visit and sex
Model 2: Adjusted for age at visit, sex and birth weight
Model 3: Adjusted for age at visit, sex and breastfeeding duration.

Table 3: Subcutaneous fat mass measured by skinfold thickness in 695 children by *FTO* genotype.

	TT (n=279)	TA (n=316)	AA(n=100)	p-value* Model 1	p-value* Model 2	p-value* Model 3
Peripheral fat mass (mm)	14.3 (3.8)	14.4 (3.9)	14.2 (3.2)	ns	ns	ns
Central fat mass (mm)	12.8 (3.5)	12.4 (3.3)	12.5 (3.2)	ns	ns	ns
Total fat mass (mm)	27.1 (6.4)	26.8 (6.3)	26.8 (5.6)	ns	ns	ns

Values represent means with standard deviation.
** p-values assume additive models.*
ns: not significant

Model 1: Adjusted for age at visit and sex
Model 2: Adjusted for age at visit, sex and birth weight
Model 3: Adjusted for age at visit, sex and breastfeeding duration.

would still have been significant. Nonetheless, it could be hypothesized that the *FTO* genotype of either child or mother is already altering body composition in early life of the child. Therefore, replication would be necessary to determine whether *FTO* really has an effect on body composition at birth or just thereafter. In another large cohort study in over 5,000 children at the age of 9 years by Frayling *et al*. a strong association (14% increase across the three genotypes) was found between the A-allele and increased fat mass, while a much weaker (1% increase across the three genotypes) effect of the A-allele was found on lean body mass.[11] Also, this study demonstrated in over 11,000 children that there was no association between this polymorphism and birth weight.

The lack of association of the *FTO* genotype with body composition at 6 months, together with the findings of Frayling *et al*., would be in line with the hypothesis that the effect of *FTO* genotype increases during childhood and therefore is not yet present in infancy. This idea was nicely demonstrated in a twin study by Haworth *et al*. where they showed that the explained variance of *FTO* on body mass index increased from less than 0.001 at the age of 4 years to 0.01 at 11 years.[12] A number of studies have examined the effect of *FTO* on a variety of phenotypes at different ages during childhood. Timpson *et al*. demonstrated in a large cohort of over 3,500 children aged 10-11 years that subjects

Table 4: Body composition measured by DXA-scan at age 6 months in 216 children by *FTO* genotype.

	TT (n=87)	TA (n=99)	AA(n=30)	p-value* Model 1	p-value* Model 2	p-value* Model 3
Fat Mass						
Truncal (grams)	675 (183)	652 (213)	652 (215)	ns	ns	ns
Peripheral (grams)	971 (247)	941 (307)	968 (263)	ns	ns	ns
Total (grams)	1964 (435)	1906 (526)	1945 (504)	ns	ns	ns
Lean Mass						
Truncal (grams)	2997 (328)	2954 (277)	2940 (336)	ns	ns	ns
Peripheral (grams)	1918 (294)	1885 (305)	1907 (286)	ns	ns	ns
Total (grams)	6484 (684)	6398 (587)	6443 (697)	ns	ns	ns
Total Mass						
Truncal (grams)	3672 (398)	3606 (397)	3592 (465)	ns	ns	ns
Peripheral (grams)	2889 (484)	2826 (553)	2875 (485)	ns	ns	ns
Total (grams)	8448 (936)	8304 (950)	8389 (1039)	ns	ns	ns
Fat Percentage						
Truncal (%)	18.3 (4.0)	17.8 (4.4)	17.9 (4.4)	ns	ns	ns
Peripheral (%)	33.3 (4.5)	32.7 (5.4)	33.3 (5.0)	ns	ns	ns
Total (%)	23.1 (3.6)	22.7 (4.3)	23.0 (4.0)	ns	ns	ns
Truncal / Peripheral Ratio	0.55 (0.07)	0.54 (0.07)	0.53 (0.06)	ns	ns	ns

Values represent means with standard deviation.
** p-values assume additive models.*
ns: not significant

Model 1: Adjusted for age at visit and sex
Model 2: Adjusted for age at visit, sex and birth weight
Model 3: Adjusted for age at visit, sex and breastfeeding duration.

with the variant A-allele had a higher fat intake (1.5 grams/day per allele) and energy intake (25kJ/day per allele).[7] In the same line, Wardle *et al*. found that children age 8-11 with the A-allele had diminished satiety, which may increase the risk of obesity.[8] In a much smaller study of 4-5 year old children, Wardle *et al*. found that children with the TT genotype also ate less at this age.[13] In a study on energy balance and expenditure in children 4-10 years of age, it was demonstrated that the A-allele was associated with increased energy intake (independent of body mass index), while not effecting energy expenditure.[10] All these studies indicate that the effect of *FTO* genotype on body composition appears to be mediated through energy intake. During breastfeeding the infant determines, in part, the amount of breast-milk (energy) intake. Restriction in our analyses to infants who were exclusively breast during the first 2 months did not change our results (data not shown). However, the negative findings in our study could be due to a lack of power. To our knowledge there are no studies on *FTO* and energy in subjects younger than 4 years of age. It could be hypothesized that *FTO* does not start to affect body composition until the children determine their energy intake themselves and that

therefore we were not able to find any effect at the age of 6 months. Studies on eating behavior and/or energy intake at younger ages will allow us to assess at what age *FTO* genotype might start having an effect.

In summary, we observed no association between the *FTO* genotype (rs9939609) and body composition, measured both by anthropometrics and DXA, at the age of 6 months. These findings appear to be consistent with the hypothesis that the effect on adiposity of *FTO* increases during childhood. Longer follow-up studies are necessary to examine at which age *FTO* genotype starts to influence fat mass, body composition and energy intake. Furthermore, systematic searches for adiposity-related common variants by means of genome-wide association study will enable us to identify other genes which influence body composition in early childhood.

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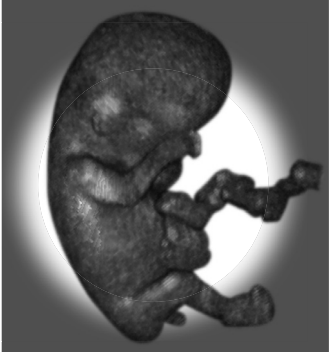

CHAPTER 3.2

Relationship between *FTO* and body mass index from birth to adolescence

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Abstract

Background: An age-dependent association between the same variant and BMI in children has been suggested, but existing studies lack dense growth data on large samples to explore association with the complex BMI trajectory at young ages.

Methods: We meta-analyzed associations between variation at the *FTO* locus (rs9939609) and BMI in samples aged from 2 weeks to 13 years from eight cohorts of European ancestry (N>19,000). After age-stratified cross-sectional analysis, we applied a mixed effects model and an extension of this to fit growth curves in infancy and childhood. We also explored association with infant adiposity peak (AP) and childhood adiposity rebound (AR).

Results: In cross-section, we identified a positive association between additional minor (A) alleles and BMI from 5.5 years onwards. In contrast, an inverse association was observed below age 2.5 years. In longitudinal analysis, the same variant was associated with lower BMI in infancy (AA vs. TT -0.11 kg/m^2 (95% CI: -0.20, -0.03), p=0.007), higher BMI in childhood (AA vs. TT 0.13 kg/m^2 (95% CI: 0.06, 0.20), p=9e^{-05}), and faster BMI growth rate in childhood (AA vs. TT 0.039 kg/m^2/year (95% CI 0.028, 0.051), p=7e^{-12}). The A-allele also associated with higher average BMI at AR (AA vs. TT 0.93 % (95%CI: 0.22, 1.64), p=0.01) and earlier AR (AA vs. TT -0.27 years (95%CI: -0.36, -0.19), p=1e^{-10}.

Conclusions: This study confirms the expected association between variation at rs9939609 and BMI in childhood, but only after an inverse association between the same variant and BMI in infancy. The positive association between rs9939609 and BMI in childhood was manifest in both differential rates of BMI change and points of inflection in modelled BMI curves. The age-dependent nature of association between rs9939609 and BMI provides important information about longitudinal gene effects and specifically about the role of *FTO* in adiposity.

Introduction

Genome-wide association studies on body mass index (BMI) and adiposity have reliably identified association with variation at the fat mass and obesity related locus (*FTO*) in adult and child populations.[1-5] In meta-analyses, the addition of each minor (A) allele at the single nucleotide polymorphism (SNP) rs9939609 within the first intron of *FTO* has been shown to be associated with a higher BMI of up to 0.33 kg/m^2 or approximately 0.1 standard deviations.[2,6] The biological mechanisms behind this association are yet to be fully determined, however evidence from both population based analyses and functional investigations have suggested that this locus is likely involved in the hypothalamic regulation of appetite or energy expenditure and metabolic rate.[7-11]

Until recently, most replication efforts concentrating on variation in *FTO* have employed cross-sectional data. These have been conducted from ages as low as 2 weeks to late age (>70 years) and have demonstrated, with differing degrees of reliability, associations between variation at *FTO*, BMI and related traits.[12,13] Relatively few investigations have focused on the nature of genetic association across the life-course through the use of longitudinal data and those that have are limited in size. Despite this, evidence that is available suggests that the cross-sectional *FTO*/BMI association does appear to differ according to age.[14-16] Specifically, at early ages up to and around 7 years, the association between common variation at *FTO* and BMI appears to be reduced in magnitude, with smaller studies being unable to detect association.[14-16] This pattern then changes into early adulthood with peak effect sizes (approximately 0.3 kg/m^2) for the association between variation at *FTO* and BMI occurring by age 20.[14,16] Following this peak, this association appears to diminish absolutely (not relatively) in a manner one would expect for coincident reduction in adiposity levels with later age.[13]

Haworth et al. simultaneously examined the *FTO*/BMI association and the heritability of BMI in a longitudinal twin collection with data at ages 4, 7, 10 and 11 years.[15] This work demonstrated that the association between BMI and variation at *FTO* appeared age dependent, that the heritability for BMI increased with age and that the proportion of variance in BMI explained by shared environment diminished over the same time period. Consistent with the idea that BMI and adiposity related traits may be determined by a complex interplay between genetic and environmental features, these findings suggest that with age and likely individual dietary autonomy, loci such as *FTO* may be able to exert a greater effect on BMI through growth and development. A Finnish twin study has since suggested an increase in the heritability of BMI throughout adolescent years.[17]

Despite the published work on longitudinal differences in the associations between the variation at *FTO* and BMI or related traits, lack of dense lifecourse data and inadequate statistical power has made interpretation of findings difficult. In this investiga-

tion, we aimed to assess the relationship between variation at rs9939609 and changes in BMI from after birth until 13 years of age, by meta-analysing data from eight cohorts consisting of over 19,000 children. Fitting individual growth curves enabled us to explore not only the association between rs9939609 and the level and the slope of the BMI curve, but also the relationship of this variant to critical change points in BMI; all features known to be related to later life health.[18-22]

Methods

Study design and cohorts

To examine the association between rs9939609 genotype and BMI from birth to 13 years of age we used the growth measurements from eight studies (Table 1). All subjects were

Table 1: Numbers and characteristics of subjects by cohort and age strata; values represent means and standard errors.

Age Stratum (years)	Cohort	N	Age (years)	Weight (kg)	Height (cm)	BMI (kg/m^2)	Boys (%)
0 - 0.49*	ALSPAC	6512	0.15 (3.6*10^{-4})	5.053 (0.0083)	57.67 (0.031)	15.16 (0.018)	50.9
	BCG	567	0.24 (1.3*10^{-3})	6.08 (0.03)	60.50 (0.10)	16.61 (0.06)	54.0
	GENR	2545	0.16 (1.6*10^{-3})	5.30 (0.02)	57.88 (0.07)	15.80 (0.03)	50.5
	NFBC1966	2954	0.26 (1.4*10^{-3})	6.15 (0.02)	61.61 (0.06)	16.20 (0.03)	49.4
	UBC	569	0.25 (1.0*10^{-3})	6.13 (0.03)	61.30 (0.10)	16.32 (0.06)	51.1
	Stratum total	13185					
0.5 - 1.49	ALSPAC	6402	0.80 (1.41*10^{-3})	9.27 (0.014)	72.65 (0.04)	17.53 (0.02)	50.7
	BCG	566	1.00 (1.5*10^{-3})	10.30 (0.05)	74.87 (0.11)	18.38 (0.07)	54.1
	GENR	2760	0.94 (2.3*10^{-3})	9.67 (0.02)	74.69 (0.06)	17.33 (0.02)	50.6
	NFBC1966	3461	0.99 (1.8*10^{-3})	10.14 (0.02)	75.52 (0.05)	17.78 (0.03)	49.3
	RAINE	1012	1.14 (2.9*10^{-3})	10.25 (0.04)	77.48 (0.09)	17.08 (0.04)	52.2
	UBC	571	1.00 (3.0*10^{-3})	10.11 (0.05)	75.92 (0.12)	17.54 (0.06)	51.3
	Stratum total	14811					
1.5 - 2.49	ALSPAC	4622	1.72 (3.0*10^{-3})	11.96 (0.02)	84.13 (0.057)	16.88 (0.022)	51.2
	BCG	548	1.99 (5.7*10^{-3})	12.72 (0.06)	85.52 (0.14)	17.39 (0.06)	54.2
	GENR	2395	2.02 (4.0*10^{-3})	12.69 (0.03)	87.69 (0.08)	16.50 (0.03)	50.3
	NFBC1966	2585	1.98 (3.1*10^{-3})	11.87 (0.02)	84.03 (0.06)	16.81 (0.02)	49.6
	RAINE	326	2.14 (6.7*10^{-3})	12.88 (0.08)	89.91 (0.20)	15.94 (0.07)	50.3
	UBC	483	1.81 (1.2*10^{-2})	12.49 (0.07)	85.97 (0.17)	16.90 (0.06)	52.6
	Stratum total	10990					
2.5 - 3.49	ALSPAC	735	2.59 (7.1*10^{-4})	13.95 (0.06)	91.56 (0.12)	16.61 (0.047)	51.6
	BCG	548	3.01 (4.8*10^{-3})	14.65 (0.07)	94.08 (0.16)	16.55 (0.06)	53.3

Table 1: Continued

Age Stratum (years)	Cohort	N	Age (years)	Weight (kg)	Height (cm)	BMI (kg/m²)	Boys (%)
	GENR	787	2.60 (3.0*10^{-3})	13.97 (0.06)	92.97 (0.13)	16.16 (0.04)	50.6
	NFBC1966	2159	3.02 (4.1*10^{-3})	14.33 (0.04)	94.48 (0.09)	16.06 (0.03)	48.3
	RAINE	733	3.09 (3.4*10^{-3})	14.98 (0.07)	96.36 (0.14)	16.13 (0.05)	50.6
	UBC	503	3.03 (4.5*10^{-2})	15.43 (0.08)	96.75 (0.17)	16.49 (0.06)	51.1
	Stratum total	5474					
3.5 - 4.49	ALSPAC	4794	3.72 (2.8*10^{-3})	16.44 (0.03)	100.44 (0.06)	16.26 (0.02)	50.7
	BCG	547	4.01 (5.6*10^{-3})	16.75 (0.08)	101.13 (0.17)	16.38 (0.05)	53.6
	NFBC1966	2122	4.02 (4.2*10^{-3})	16.23 (0.04)	101.71 (0.09)	15.69 (0.03)	46.9
	UBC	525	4.04 (4.2*10^{-3})	17.65 (0.09)	104.53 (0.19)	16.15 (0.06)	51.6
	Stratum total	8023					
4.5 - 5.49	ALSPAC	679	5.17 (2.3*10^{-3})	19.46 (0.09)	110.27 (0.17)	15.96 (0.05)	51.5
	BCG	560	5.00 (3.2*10^{-3})	18.71 (0.09)	107.32 (0.18)	16.24 (0.06)	53.6
	NFBC1966	1901	5.02 (4.5*10^{-3})	18.11 (0.05)	108.37 (0.11)	15.42 (0.03)	46.3
	UBC	151	5.13 (2.6*10^{-2})	20.08 (0.26)	111.83 (0.42)	16.05 (0.13)	51.0
	Stratum total	3299					
5.5 – 6.99	EBS	714	6.44 (1.4*10^{-2})	23.39 (0.14)	118.75 (0.20)	16.59 (0.06)	50.3
	NFBC1966	2759	6.34 (6.6*10^{-3})	20.86 (0.06)	116.62 (0.10)	15.33 (0.03)	48.9
	RAINE	984	5.90 (5.7*10^{-3})	21.16 (0.09)	116.00 (0.15)	15.73 (0.05)	52.2
	UBC	500	6.02 (2.2*10^{-2})	22.19 (0.15)	118.14 (0.26)	15.90 (0.07)	50.4
	Stratum total	4957					
7 – 9.99	ALSPAC	5549	7.83 (6.3*10^{-3})	26.72 (0.06)	127.49 (0.08)	16.35 (0.03)	50.8
	EBS	1350	8.02 (1.6*10^{-2})	27.97 (0.14)	127.90 (0.17)	17.09 (0.06)	49.2
	NFBC1966	3261	7.82 (6.7*10^{-3})	24.33 (0.06)	124.60 (0.10)	15.68 (0.03)	50.2
	RAINE	986	8.10 (1.0*10^{-2})	27.82 (0.16)	129.34 (0.18)	16.63 (0.07)	52.0
	UBC	510	7.84 (2.3*10^{-2})	27.23 (0.23)	129.14 (0.28)	16.29 (0.09)	51.6
	Stratum total	11694					
9 – 10.99	ALSPAC	5159	9.94 (4.6*10^{-3})	34.63 (0.10)	139.73 (0.09)	17.63 (0.04)	49.9
	CH	545	10.26 (1.4*10^{-2})	33.19 (0.17)	138.72 (0.23)	17.25 (0.06)	100
	NFBC1966	2602	10.06 (8.2*10^{-3})	31.43 (0.11)	137.43 (0.13)	16.64 (0.04)	50.6
	RAINE	945	10.57 (4.6*10^{-3})	37.87 (0.26)	143.68 (0.21)	18.34 (0.10)	52.4
	UBC	510	10.51 (2.0*10^{-2})	35.36 (0.31)	142.22 (0.30)	17.48 (0.11)	51.6
	Stratum total	9798					
11 - 13	ALSPAC	4635	11.75 (3.3*10^{-3})	43.26 (0.14)	150.63 (0.11)	18.94 (0.05)	49.3
	CH	811	12.01 (3.9*10^{-3})	38.67 (0.17)	146.63 (0.21)	17.99 (0.05)	100
	NFBC1966	3389	11.88 (6.2*10^{-3})	37.99 (0.12)	147.32 (0.13)	17.50 (0.04)	50.3
	UBC	339	12.03 (2.7*10^{-2})	43.47 (0.49)	153.46 (0.44)	18.45 (0.15)	52.2
	Stratum total	9203					

** Birth weight and height were excluded from analyses.*

Subjects are all singletons of Caucasian ethnicity with FTO (rs9939609) genotype and weight/height information available. No siblings have been included.

unrelated children of white European ancestry. All multiple births were excluded from the analyses. When multiple siblings were present in a specific cohort, only data from the oldest sibling was used for analyses. All studies have previously been described in detail, but a brief description of each study is shown below.

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective birth cohort in Bristol, UK, which recruited pregnant women with expected delivery dates in 1991-1992 (present analysis: 7,482 subjects). ***The Barry Caerphilly Growth Study*** (BCG) is a longitudinal study of infants born in the towns of Barry and Caerphilly in South Wales between 1972 and 1974 (569 subjects). ***The Christ's Hospital Cohort*** (CH) is a retrospective follow-up study comprised of former male students between the ages of 10 and 18 years of the Christ's Hospital born between 1927 and 1956 (812 subjects). As part of the ***Energy Balance Study*** (EBS), data was collected in 2002 and 2003 on pre-pubertal schoolchildren, ages 4 through 10 years, from north-eastern Scotland (2,604 subjects). ***The Generation R Study*** (GENR) is a prospective birth cohort from early foetal life onwards based in Rotterdam, the Netherlands; subjects born between 2002 and 2006 (2,851 subjects). ***The Northern Finland Birth Cohort 1966*** (NFBC1966) is a prospective pregnancy/birth cohort with expected deliveries in 1966 in the two northernmost provinces of Finland (3,707 subjects). ***The Raine Study*** (RAINE) is a prospective pregnancy cohort set up in 1989, which recruited pregnant women from Perth, Western Australia for ultrasound imaging (1,106 subjects). The ***Uppsala Birth Cohort Multigeneration Study*** (UBC), is a multigenerational study based set up in 1995 in Uppsala, Sweden (594 subjects).

In all studies, growth characteristics (weight, height) were measured during routine visits at community health centres or at the research centres. All subjects (or their parents/guardians) gave an informed consent and each study obtained ethical approval from the local ethical review board. Genotyping of the rs9939609 was performed directly in all eight cohorts. DNA was isolated either from buccal swabs, blood or cord blood..

Analytical strategy and statistical analysis

Cross-sectional analyses

Based on the ages at which the subjects visited the community health or research centres in the various studies, growth measurements were grouped into ten strata: 0.01 to 0.49 years (i.e. excluding birth); 0.50 to 1.49 years; 1.50 to 2.49 years; 2.50 to 3.49 years; 3.50 to 4.49 years; 4.50 to 5.49 years; 5.50 to 6.99 years; 7.00 to 8.99 years; 9.00 to 10.99 years; and 11.00 to 12.99 years. To approximate normality in each study, BMI was $\log_e$ transformed and stratum-specific Z-scores were created. All values with a Z-score values above or below ± 3.0 were removed. To examine the association between *FTO* genotype and BMI, defined as weight[kg]/(height[m])^2, within each age stratum, we used multi-

variable linear regression.[23] Study-specific effect estimates within each age-stratum were created assuming an additive genetic model. These models were adjusted for age because age varied within each age-stratum and sex. The use of further covariates was restricted as is has been shown that variation at rs9939609 is not associated with either birth weight or gestational age[2] and the distribution of genotypes is assumed to be unrelated to possible environmental confounders.[24] To take into account known correlations between BMI and height at early ages, analyses were repeated in a further sensitivity analysis using weight/height^p (p ranging from 1.7 to 2.8), where we created stratum- and sex-specific p scores for each study.[25] Basic analyses were performed in Stata11 (Stata corp.). Cross-sectional cohort specific analysis results were meta-analyzed (inverse variance) within each stratum using a fixed effects model if variance was homogeneous (all age strata under 9 years of age) and random effects model if variance was heterogeneous (9-13 years using the "Rmeta" package in R (version 2.6.2).

Longitudinal analyses

Modelling BMI longitudinally is complex due to the early adiposity peak in infancy and the increasing variance in BMI measurements between individuals throughout childhood. Polynomial curves have generally a poor fit when BMI is modelled from birth until age 13 years and they overestimate the timing of the adiposity peak and rebound. To overcome these complexities, statistical modelling was split into two age windows; 2 weeks to 18 months (infancy) and 18 months to 13 years (childhood), using the studies that contained the most data within these age windows (BCG, Generation R, NFBC1966 and Uppsala in the 2 weeks to 18 months window and ALSPAC, RAINE, NFBC1966 and Uppsala in the 18 months to 13 years window). BMI measurements below 2 weeks of age were excluded due to the natural reduction in body weight immediately after birth. The 18 month change point between infancy and childhood was chosen on both biological and statistical grounds. In both age windows, cohort specific analysis results for both intercept (baseline BMI) and slope (change in BMI over time) were meta-analyzed (inverse variance) using a fixed effects model in the "Rmeta" package in R (version 2.6.2).

Longitudinal analyses in infancy (2 weeks to 18 months):

Linear mixed effects (LME)[26] models for BMI were fitted using sex, genotype, gestational age and their interactions with age as covariates, with random effects for intercept (baseline BMI) and slope (linear change in BMI over time). In addition to the linear age effect, quadratic and cubic terms for age were included to account for nonlinearity of BMI change over time. The model is written as:

$$\text{BMI (kg/m}^2) = \beta_0 + \beta_1 \text{ Age} + \beta_2 \text{ Age}^2 + \beta_3 \text{ Age}^3 + \beta_4 \text{ Sex} + \beta_5 \text{ Gest\_age} + \beta_6 \textit{FTO} \text{ TA genotype} + \beta_7 \textit{FTO} \text{ AA genotype} + \beta_8 \text{ Age * Sex} + \beta_9 \text{ Age * TA genotype} + \beta_{10} \text{ Age * AA genotype} + \beta_{11} \text{ Age * Gest\_age} + u_0 + u_1 \text{ (Age)} + \varepsilon$$

Where: β_n are the fixed effects, u_n are the individual level random effects and ε is the residual error. The age component was centred to 9 months of age and gestational age (Gest_age) to 40 weeks before fitting the model. Several sensitivity analyses were also conducted to ensure that the inferences from the above model wouldn't change if different parameters were incorporated. These included changing the upper limit of age from 18 months to 12 or 24 months, adjusting for BMI at birth, birth weight and duration of breast feeding in early life (where available), inclusion of BMI measurements from the first two weeks of life, and removing the adjustment for gestational age.

Longitudinal analyses in childhood (beyond 18 months to 13 years):

Beyond 18 months, an extension to the linear mixed effects (LME) model based on multivariate skew *t* distribution was applied to provide robustness against the normality assumptions of random effects and random error in the LME framework. This multivariate skew-*t* model[27,28] assumes the random effects follow a multivariate skew-normal distribution while the subject measurement errors follow a *t* distribution (BMI was not log-transformed in these analyses). The same covariates and random effects were chosen as in the infancy analysis, except that this analysis was not adjusted for gestational age. Age was centred at 7 years 3 months before fitting the model. Sensitivity analysis on the additional adjustment for baseline BMI at 18 months of age was performed to ensure stability of inferences. Additionally, a comparison was made between the skew-*t* model and a LME model to determine the best fit for the data.

Derivation of age and BMI at adiposity peak (AP) and adiposity rebound (AR):

Age and BMI at AP and AR were derived from models similar to those described above. The same age thresholds (2 weeks – 18 months and >18 months – 13 years) were used. The LME model was chosen for this analysis since it gave nearly identical estimated values for the growth parameters as the skew-t model (correlation coefficients = 0.93-0.99 in RAINE and Uppsala), but was more efficient. Linear, quadratic and cubic age effects and sex effect were included. rs9939609 genotype was removed from these models since we wanted to estimate age and BMI at AP and AR independently and to then analyse their association with *FTO*. Subsequently, sex interactions with linear and quadratic age effects were added in the childhood model (for both interactions $p<0.01$ in ALSPAC and in the NFBC1966). Random terms for the intercept and slope were included in both models to allow individual departures from the common intercept and slope. The models are written as:

(1) Infancy model:

$$\log(\text{BMI (kg/m}^2)) = \beta_0 + \beta_1 \text{ Age} + \beta_2 \text{ Age}^2 + \beta_3 \text{ Age}^3 + \beta_4 \text{ Sex} + u_0 + u_1 \text{ (Age)} + \varepsilon$$

(2) Childhood model:

$$\log(\text{BMI (kg/m}^2)) = \beta_0 + \beta_1 \text{ Age} + \beta_2 \text{ Age}^2 + \beta_3 \text{ Age}^3 + \beta_4 \text{ Sex} + \beta_5 \text{ Age} * \text{Sex} + \beta_6 \text{ Age}^2 * \text{Sex} + u_0 + u_1 \text{ (Age)} + \varepsilon$$

For each participant, a predicted BMI at AP and AR (at a minimum resolution of every 0.05 years in infancy and every 0.1 years in childhood) was calculated using the estimated fixed and random coefficients. Age at AP was defined as the age at maximum BMI between 0.25 and 1.25 years and age at AR as the age at minimum BMI between 2.5 and 8.5 years. These cut-off points were chosen based on descriptive analysis of growth curves in the NFBC1966. The associations between rs9939609 genotype and these new growth parameters were analyzed using additive genetic models. To account for uncertainty in the derived parameters, each person's data were weighted by the number of measurements within the age window in the association analyses. Persons who had fewer than three measurements per age window were excluded from the analyses, since capturing the complex relationship between age and BMI becomes impossible with fewer than three measurement points. Sensitivity analyses with gestational age as a further adjustment in the AP models made no substantive differences to the results (performed in the NFBC1966), and since this adjustment would have decreased numbers in some of the cohorts, it was omitted. Age at AP and age at AR were analysed without any transformation. The distribution of BMIs at AP and AR were somewhat skewed to the right - therefore log-transformed - and association results are reported as percentage differences in BMI between genotypes.

In both age windows, cohort specific association results between each growth parameter and *FTO* were meta-analyzed (inverse variance) using a fixed effects model in the "Rmeta" package in R (version 2.6.2) and in parallel with the MetaAnalyst (Beta 3.13). In case of heterogeneity, results from a random effects model were reported.

Results

Cross-sectional results

Table 1 describes the stratum and study-specific subject characteristics. Figures 1a-d and figure 2 show the results of the meta-analyses of the association between each additional minor allele (A) at rs9939609. In meta-analyses above the age of 5.5 years the minor allele (A) was associated with a higher BMI, though this was not detectable in the age stratum 11 to 13 years where the sample was small. The additive effect of each

Figure 1a: Metaplot of the effect of *FTO* genotype on logBMI age 0.5-1.5 years.

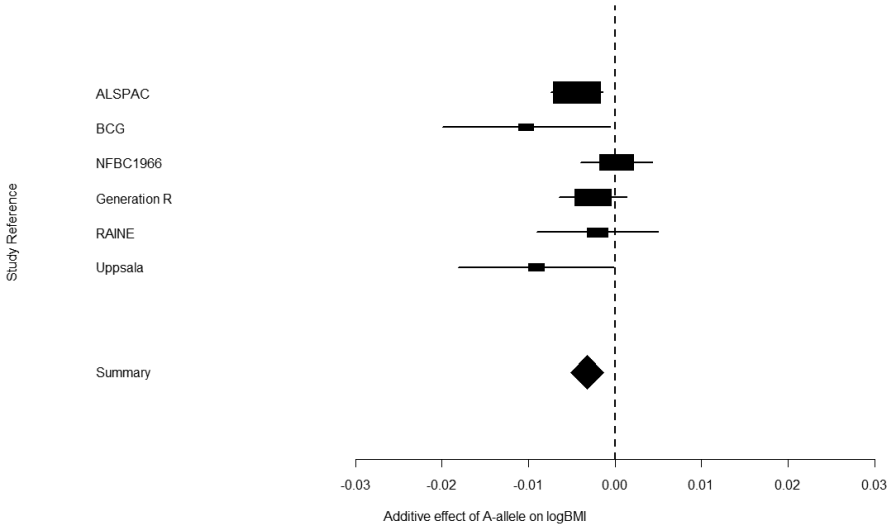
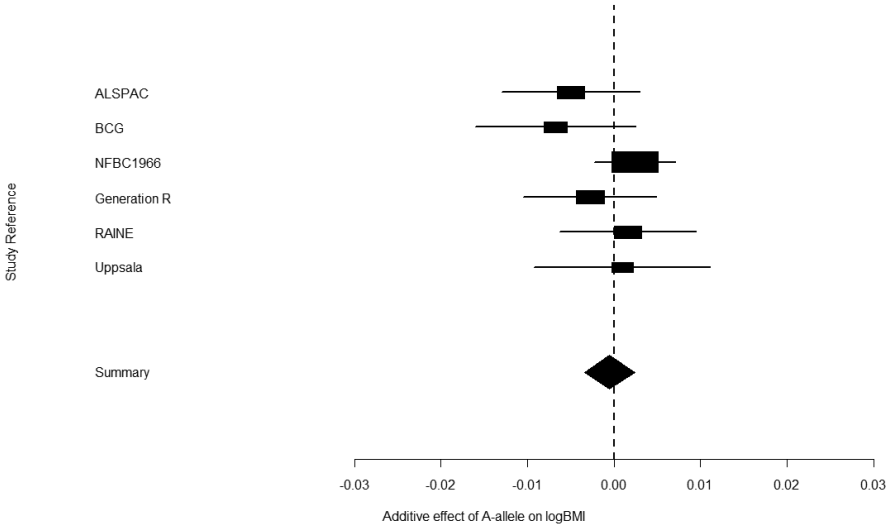


Figure 1b: Metaplot of the effect of *FTO* genotype on logBMI age 2.5-3.5 years.



minor allele (A) was 0.26 kg/m^2 (95% CI: 0.13, 0.39 kg/m^2), 0.35 kg/m^2 (95% CI: 0.25, 0.46 kg/m^2), 0.53 kg/m^2 (95% CI: 0.24, 0.46 kg/m^2) and 0.32 kg/m^2 (95% CI: -0.09, 0.73 kg/m^2) at 5.5-7 years, 7-9 years, 9-11 years and 11-13 years, respectively. Justifying the use of a random effects model, maximum heterogeneity in this meta-analysis was high and reflected in an I^2 value of 70% (95% CI: 22%, 88%).

Figure 1c: Metaplot of the effect of *FTO* genotype on logBMI age 5.5-7 years.

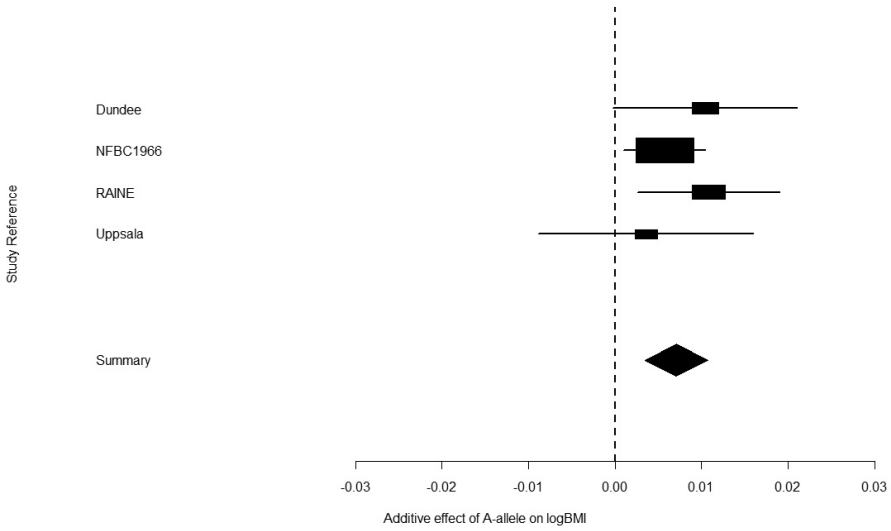
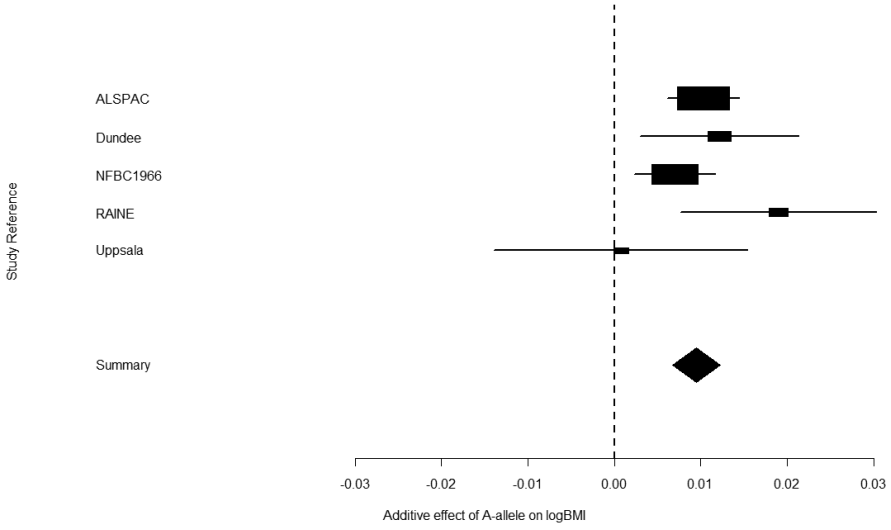
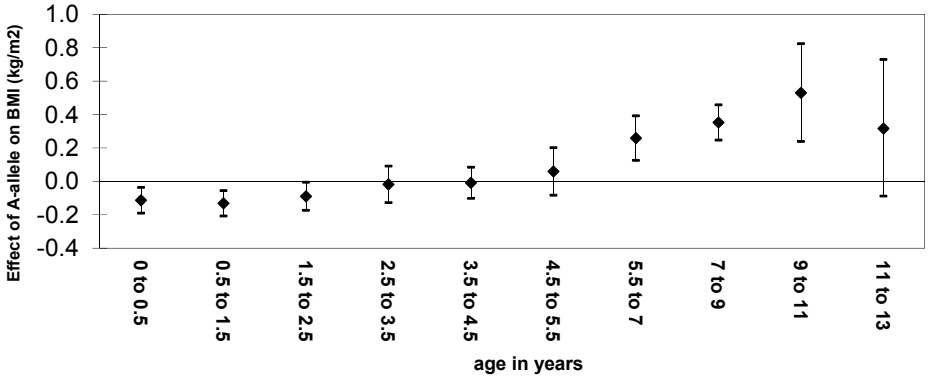


Figure 1d: Metaplot of the effect of *FTO* genotype on logBMI age 7-9 years.



In contrast to this, each minor allele was associated with a *lower* BMI before the age of 2.5 years. The additive effect of each minor allele was -0.11 kg/m^2 (95% confidence interval (CI): -0.19, -0.04 kg/m^2) at age 0-0.5 years, -0.13 kg/m^2 (95% CI: -0.21, -0.06 kg/m^2) at 0.5-1.5 years, and -0.09 kg/m^2 (95% CI: -0.17, -0.01 kg/m^2) at 1.5-2.5 years. Between the ages of 2.5 and 5.5 years, no cross-sectional association was observed between

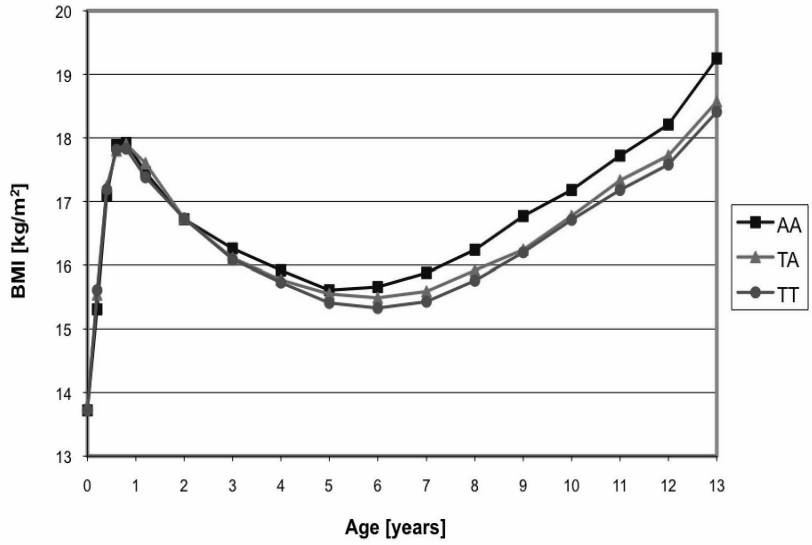
Figure 2: Summary of the association between rs9939609 minor allele (A) and body mass index per age stratum.



Effect estimates are derived by back transformation of the effects from the meta-analyses. Maximum heterogeneity in meta-analyses was I^2 82 (95% CI: 53, 93).

rs9939609 genotype and BMI. Similar results (not shown) were found for analyses regarding weight/height[p]. Again, maximum heterogeneity in these analyses was high and reflected in an I^2 value of 82 (95% CI: 53%, 93%). These patterns can be crudely summarised by plotting mean BMI by genotype at different ages in the NFBC1966 (the single cohort with the most dense data from birth to 13 years of age) (Figure 3).

Figure 3: Observed mean BMI at 0-13 years of age in the NFBC1966 by the *FTO* SNP rs9939609 (minor (A) allele adiposity associated).



Mean values are calculated (not predicted) from the NFBC1966 (the cohort with most dense data from birth to 13 years of age).

Longitudinal results

Derived from longitudinal infancy analysis, the rs9939609 minor allele (A) was associated with a lower estimated mean BMI at the centre point of 9 months of age; difference in BMI between genotypes AA (two risk alleles) and TT was -0.11 kg/m^2 (95%CI: -0.20, -0.03), p=0.007 (Table 2). In contrast, in the childhood analysis, the risk allele was associated with a higher BMI at the centre point of 7.25 years; difference in BMI between genotypes AA and TT was 0.13 kg/m^2 (95%CI: 0.07, 0.20), p=9e^{-5}. Heterogeneity in these

Table 2: Differences in body mass index by *FTO* genotype. Reference group: genotype TT.

Genotype	Study	Difference in BMI (kg/m^2)	
		0 to 1.5 years	**1.5 to 13 years**
TA	ALSPAC	N/A	0.05 (0.04), p=0.1
	BCG	0.01 (0.12), p=0.9	N/A
	GENR	-0.06 (0.04), p=0.2	N/A
	RAINE	N/A	0.13 (0.09), p=0.1
	NFBC1966	0.02 (0.05), p=0.7	0.05 (0.04), p=0.2
	UBC	-0.14 (0.11), p=0.2	-0.10 (0.12), p=0.4
	Meta-analysis	-0.04 (95% CI: -0.09, 0.03), p=0.3	0.05 (95% CI: 0.00, 0.10), p=0.04
AA	ALSPAC	N/A	0.15 (0.05), p=0.002
	BCG	-0.32 (0.17), p=0.05	N/A
	GENR	-0.13 (0.06), p=0.03	N/A
	RAINE	N/A	0.23 (0.13), p=0.08
	NFBC1966	-0.05 (0.07), p=0.5	0.11 (0.05), p=0.04
	UBC	-0.13 (0.14), p=0.4	0.01 (0.16), p=0.9
	Meta-analysis	-0.11 (95% CI: -0.20, -0.03), p=0.007	0.13 (95% CI: 0.07, 0.20), p=9e^{-05}

Values are regression coefficients (95% confidence interval) and represent differences in BMI (kg/m^2) at centre points of age: 0.75 years in infancy and 7.25 years in childhood.
Heterogeneity was not detected in the meta-analyses.
NA denotes insufficient data for longitudinal analysis.

analyses was low and I^2 statistics did not exceed 0% in either case.

The rate of change in BMI was associated with variation at rs9939609 locus in childhood but not in infancy. BMI increased faster in those with two minor (A) alleles compared to the reference (TT) group. This equated to a difference of 0.039 kg/m^2/year (95%CI: 0.028, 0.051) around age 7.25 years, p=7e^{-12} (Table 3). In these of BMI change, values for heterogeneity were larger (I^2 from 30-65%) and again warranted use of random effects models. Sensitivity analyses indicated that the inferences for the intercept and slope terms of rs9939609 genotype (main effect and interaction with age) were not notably affected by

Table 3: Differences in body mass index growth rate by *FTO* genotype. Reference group: genotype TT.

Genotype	Study	Difference in BMI per year (kg/m²)	
		0 to 1.5 years	**1.5 to 13 years**
TA	ALSPAC	N/A	0.030 (0.007), p=3.4e-06
	BCG	-0.097 (0.127), p=0.5	N/A
	GENR	0.015 (0.053), p=0.8	N/A
	RAINE	N/A	0.028 (0.016), p=0.08
	NFBC1966	0.132 (0.066), p=0.04	0.003 (0.006), p=0.6
	UBC	0.190 (0.109), p=0.08	0.003 (0.017), p=0.9
	Meta-analysis	0.062 (95% CI: -0.010, 0.134) p=0.092	0.016 (95% CI: 0.008, 0.024), $p=1e^{-04}$
AA	ALSPAC	N/A	0.058 (0.009), $p=1.9e^{-11}$
	BCG	-0.205 (0.177), p=0.3	N/A
	GENR	0.065 (0.074), p=0.4	N/A
	RAINE	N/A	0.027 (0.0263), p=0.3
	NFBC1966	0.098 (0.090), p=0.3	0.026 (0.0085), p=0.003
	UBC	-0.162 (0.146), p=0.3	0.016 (0.0227), p=0.5
	Meta-analysis	0.026 (95% CI: -0.074, 0.125) p=0.6	0.039 (95% CI 0.028, 0.051), $p=7e^{-12}$

Values are regression coefficients (95% confidence interval) and represent differences in BMI per year (kg/m² per year) at centre points of age: 0.75 years in infancy and 7.25 years in childhood.
In these longitudinal analyses, values for heterogeneity were I^2 from 30-65%.
NA denotes insufficient data for longitudinal analysis.

the inclusion of additional covariates (outlined in the method section) or by moving the cut-off point between the two age windows from 18 to 12 or 24 months of age.

In the analyses between rs9939609 and both age at AP/AR and BMI at AP/AR, there was weak evidence for a lower BMI at AP in the carriers of two minor alleles (AA) compared to the reference group (TT): -0.401% (95%CI: -0.741, -0.060), p=0.02 (Table 4). In contrast, by the age of AR, this effect showed evidence for inversion with carriers of two minor alleles (AA) having higher BMI at AR than those in the reference group (TT): 0.933 % (95%CI: 0.224, 1.642), p=0.01. Whilst there was no evidence for genotypic association with age at AP in the meta-analysis, there was evidence for an earlier AR in the carriers of two minor alleles (AA) compared to the reference group (TT): -0.273 years (95%CI: -0.358, -0.188), $p=1e^{-10}$. Among AP and AR analyses, there was evidence for heterogeneity between the cohorts only when age at AR was compared between carriers of one minor allele (TA) and the reference group (TT) (I^2 72% (95% CI: 21%, 90%) and when BMI at AR was compared between carriers of two minor alleles (AA) and the reference group (TT) (I^2 69% (95% CI: 12%, 89%). For consistency, we used random effects models for comparisons regarding meta-analyses on age and on BMI at AR.

Table 4: Differences in age and body mass index (BMI) at adiposity peak (AP) and adiposity rebound (AR) between *FTO* genotype groups with 95% confidence interval (95% CI). Reference group: genotype TT.

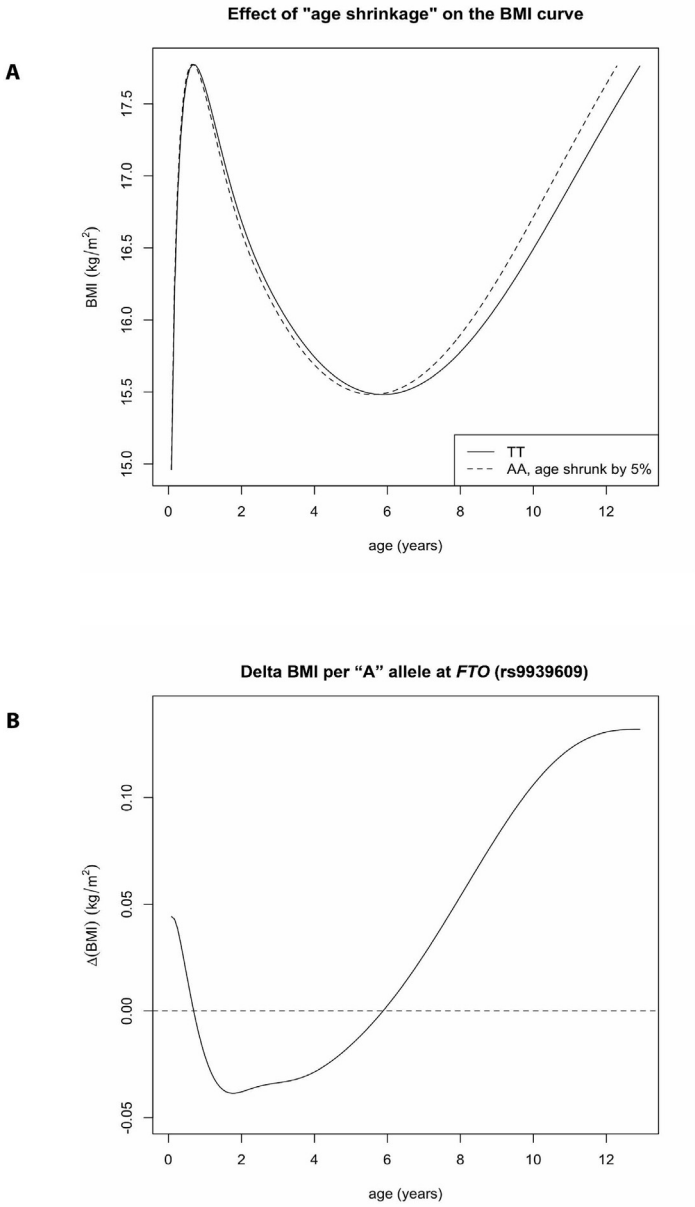
Genotype	Study	Age difference at AP [weeks]	% difference of BMI at AP
	BCG	-0.050 (-0.228, 0.129), p=0.6	0.062 (-0.852, 0.985), p=0.9
	GENR	0.003 (-0.176, 0.181), p=1	-0.226 (-0.563, 0.113), p=0.2
TA	NFBC1966	-0.054 (-0.306, 0.199), p=0.7	0.113 (-0.384, 0.613), p=0.7
	UBC	0.176 (0.009, 0.343), p=0.04	-0.495 (-1.111, 0.126), p=0.1
	Meta-analysis	**0.035 (-0.059, 0.128), p=0.5**	**-0.164 (-0.411, 0.082), p=0.2**
	BCG	-0.078 (-0.328, 0.172), p=0.5	-1.368 (-2.627, -0.092), p=0.04
	GENR	-0.019 (-0.270, 0.233), p=0.9	-0.505 (-0.977, -0.030), p=0.04
AA	NFBC1966	0.273 (-0.069, 0.615), p=0.1	-0.260 (-0.930, 0.415), p=0.5
	UBC	-0.131 (-0.355, 0.093), p=0.3	0.103 (-0.725, 0.938), p=0.8
	Meta-analysis	**-0.030 (-0.159, 0.099), p=0.7**	**-0.401 (-0.741, -0.060), p=0.02**
Genotype		**Age difference at AR [years]**	**%difference of BMI at AR**
	ALSPAC	-0.181 (-0.253, -0.109), p=8.0e-07	0.621 (0.157, 1.088), p=0.009
	RAINE	-0.257 (-0.405, -0.110), p=6.6e-04	0.414 (0.073, 0.756), p=0.02
TA	NFBC1966	-0.067 (-0.137, 0.003), p=0.06	0.518 (-0.010, 1.050), p=0.06
	UBC	0.051 (-0.207, 0.309), p=0.7	-0.141 (-1.480, 1.216), p=0.8
	Meta-analysis	**-0.134 (-0.234, -0.034), p=0.009**	**0.473 (0.234, 0.713), p=1e^{-04}**
	ALSPAC	-0.354 (-0.452, -0.256), p=1.6e-12	1.256 (0.620, 1.896), p=1.0e-04
	RAINE	-0.241 (-0.451, -0.030), p=0.03	0.313 (-0.172, 0.799), p=0.2
AA	NFBC1966	-0.224 (-0.319, -0.129), p=3.8e-06	1.584 (0.860, 2.314), p=1.7e-05
	UBC	-0.138 (-0.484, 0.208), p=0.4	0.266 (-1.534, 2.099), p=0.8
	Meta-analysis	**-0.273 (-0.358, -0.188), p=4e^{-10}**	**0.933 (0.224, 1.642), p=0.01**

Age and BMI at AP and AR have been estimated from the LME model for all cohorts.
Meta-analysis results (for AP parameters) are based on fixed effects models. Heterogeneity statistic was present for: Heterogeneity only present when age at AR was compared between TA and the reference group TT (I^2 72% (95% CI: 21%, 90%) and when BMI at AR was compared between AA and the reference group TT (I^2 69% (95% CI: 12%, 89%).

Discussion

We present a large, longitudinal, investigation into the association of rs9939609 with BMI. Results here not only confirm the increasing magnitude of associations between this variant and adiposity from the end of infancy through to childhood, but also suggest an inverse association at very early ages and association with the timing of key developmental events. With resolution afforded by large sample size (>19,000 children) including cohorts with dense anthropometric measurement data, we have been able to demonstrate that rather than a null association between the rs9939609 adult adiposity associated variant and BMI before the age of 2.5 years, each extra minor (A) allele at this locus appears to confer a decrease in BMI. Beyond the age of 5.5 years, anticipated

Figure 4a/4b: Longitudinal effects of rs9939609 considered as "age shrinkage" throughout growth and development.



Data are based on boys median BMI for British 1990 growth standards[29].
***A** – shows a representation of the effects of "age shrinkage" on the BMI curve by genotype at rs9939609. Curve was summarised as a natural cubic spline on the log age scale with 9 degrees of freedom, and then differentiated with respect to log age. This assumes an effect of constricting age at adiposity rebound of 5%.*
***B** – shows the per allele difference in BMI (delta BMI) attributable to an "age shrinkage" effect derived from a 5% effect on age at adiposity rebound.*

patterns of association between rs9939609 and BMI gradually emerges: in cross section, minor allele carriers show greater levels of BMI and when assessed longitudinally, they exhibit steeper rates of BMI increase throughout childhood. We have also been able to show that the minor allele is associated with a lower BMI at AP, a higher BMI at AR and an earlier timing of AR.

The changing associations between rs9939609 and BMI over the course of infancy and childhood may be consistent with what is known about the biology of this locus. If *FTO* is indeed operating through an influence on appetite and the amount of food consumed,[7,11] then it may be that until individuals are able to autonomously regulate dietary intake, the effect of variation at this locus will not be realised. This reflects an anticipated interplay between genetic and environmental factors supported also by the only simultaneous study of the heritability and genetics of BMI to date.[15]

The switch in direction of the rs9939609-BMI association from early to mid-childhood is unexpected, but a possible explanation for it is that each additional minor (A) allele at rs9939609 is associated with faster growth and development. In this case and for any given time, carriers of the A allele would be more developmentally advanced. To illustrate this, average age at AR is approximately 5.7 years for the TT genotype (Figure 3), and is 0.13 and 0.28 years earlier for TA and AA respectively (Table 4). In a simplified scheme, this may be taken as an effective "shrinking" of the AR age scale by approximately 5% when comparing TT versus AA genotypes. The effect of this hastening of development on the median BMI curve can be modelled in standard data taken from the British 1990 growth data[29] (Figure 4a) and in agreement with findings here, at ages when the BMI curve is rising the AA(fast growth) curve is raised relative to TT(slower growth) and where the BMI curve is falling the AA curve is lowered relative to TT. This effect can be quantified by differentiating the BMI curve with respect to log age and multiplying by the shrinkage factor. Figure 4b shows the putative effect per minor (A) allele of 2.5% age shrinkage. Between 1.5 and 4 years each A allele is predicted to *lower* BMI by ~0.04 units, while after age 7 each allele *increases* BMI by ~0.1 units. These effects are broadly similar to those reported in Figure 2.

As to physiological evidence supporting such an age shrinkage mechanism, rapid early weight gain is a known risk factor for later obesity[30-32] and since weight gain is calculated as weight increment divided by time interval, shortening the time interval has the same impact on gain as increasing the increment. Secondly, early puberty is known to be associated with greater obesity[33,34] and early puberty corresponds to an advanced developmental age. Furthermore it has been shown that differences between individuals in their internal body clock explain most of the variability in the pubertal height growth curve.[35]

Despite the considerable statistical gains afforded to this study through a large sample size, there are limitations to our study. Firstly, the binning of sample contributions and

definition of analytic windows in longitudinal data may not be optimal. There are still age groups for which we have limited sample size and this is reflected in the sampling error associated with these periods of growth and development. Furthermore, although the ability to examine the influence of this locus at different ages is aided by an analysis of many samples, the strata specific estimates and their error terms are subject to the different measurement techniques and ages.

Secondly, possible complication to the patterns of association seen between common variation at the *FTO* gene and BMI relates to the interplay between maternal and offspring genotypes. Whilst not formally tested here and not within the bounds this paper, the observation that mothers with greater BMI have, on average, offspring with greater birthweight may be relevant in our interpretation of results.[36-40] Owing to the correlation between maternal and foetal genotype, individuals carrying minor (A) adiposity raising alleles at rs9939609 will more be more likely to have mothers harbouring the same variants. One may hypothesise that, on average, the elevation of adiposity in these mothers may translate to increased levels of birthweight or differential growth and development in early ages as shown observationally.[41,42] Whilst this pattern fits with childhood patterns shown here, this would act to counter the inverse association between minor (A) alleles at rs9939609 and BMI at very young ages (i.e. reduce the observed negative association between rs9939609 and BMI in infancy). Confirmation of this requires further large collections with available maternal genotypes, however analysis in the ALSPAC study suggests that after adjustment for maternal genotype the negative association with BMI at young ages is present and is marginally stronger (although it is difficult to assess this association reliably without further data).

Lastly, the use of BMI as an assessment of adiposity at early ages has been disputed,[22,43] although BMI is still commonly used. In this investigation, we performed sensitivity analyses using the derived measure weight/height$^{p\,25}$ to account for this limitation and found that results were largely consistent with weight/height.[2] For this reason and for consistency with later ages in childhood, we adopted the use of BMI throughout. Overall, we conducted a large, longitudinal analysis of the association between common variation at *FTO* locus and BMI among over 19,000 children. We have noted that the effect of this locus appears to be age dependent. Importantly our results also suggest an inversion of the known adult association between this locus and BMI at ages below 2.5 years, an observation which might help develop understanding as to biological mechanisms behind the association between common variation at this locus and adiposity related traits. Further, specific, analyses will be required to confirm the associations between variation at rs9939609 and adiposity (especially at very young ages) and to investigate the clinical implications of associations between common genetic variation and both BMI at AP and the timing of AR.

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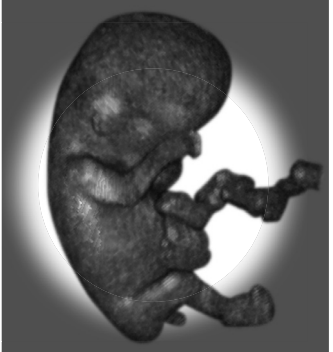

CHAPTER 3.3

Type 2 diabetes gene *TCF7L2* polymorphism and fetal and postnatal growth

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Abstract

Background: An inverse association between birth weight and the risk of developing type 2 diabetes (T2D) in adulthood has been reported. This association may be explained by common genetic variants related to insulin secretion and resistance, since insulin is the most important growth factor in fetal life. The objective of this study was to examine whether T2D gene polymorphism *TCF7L2* rs7903146 is associated with growth patterns from fetal life until infancy.

Methods: This study was performed in two independent birth cohort studies, one prospective population-based (Generation R), and one of subjects born small-for-gestational-age (SGA cohort). Fetal growth was assessed by ultrasounds in second and third trimesters of pregnancy in Generation R. Growth in infancy was assessed in both cohorts at birth and at 6, 12 and 24 months postnatally. *TCF7L2* genotype was determined in 3,419 subjects in Generation R and in 566 subjects in the SGA cohort.

Results: Minor allele frequency did not differ significantly (p=0.47) between Generation R (T-allele: 28.7%) and the SGA cohort (T-allele: 29.8%). No differences at birth were found in gestational age or size (head circumference, length, weight) between the genotypes in either cohort. *TCF7L2* genotype was also not associated with any pre- or postnatal growth characteristic in either Generation R or the SGA cohort.

Discussion: We found no evidence for an association between *TCF7L2* genotype and fetal and early postnatal growth. Furthermore, this *TCF7L2* polymorphism was not associated with an increased risk of SGA.

Introduction

Several epidemiological studies have shown inverse associations between birth weight and metabolic diseases, including type 2 diabetes (T2D) in adulthood.[1,2] These associations may be influenced by common genetic variants.[2] Insulin is the most important fetal growth factor and insulin-mediated fetal growth might be affected by genetic polymorphisms that regulate fetal insulin secretion or insulin sensitivity.[2] Therefore, gene variants associated with T2D have been suggested as candidate genes for influencing early growth.[2]

Genome-wide association (GWA) studies have consistently shown that the C>T substitution in *TCF7L2* gene *(*rs7903146) increases the risk of T2D approximately 2-fold when two risk allele copies (TT) are present.[3-5] The T-allele of this *TCF7L2* polymorphisms has been suggest to reduce proinsulin to insulin conversion,[6] though the exact mechanism has not been elucidated yet. Other single nucleotide polymorphisms (SNPs) of the *TCF7L2* gene have been shown to be associated with type 2 diabetes, although less strongly.[7] The T-allele of rs7903146, which according to HapMap has an allele frequency amongst Caucasians (CEU) of 28%,[8] has been shown to be associated with reduced insulin response and secretion in both diabetic and non-diabetic individuals,[9-11] though results in non-diabetics are not consistent.[12] This polymorphism may also lead to an increased risk of gestational diabetes.[13] Such findings make *TCF7L2* one of the most important candidate genes for explaining the associations between low birth weight and T2D.

Freathy *et al*. were the first to investigate the association between *TCF7L2* genotype and birth weight, and they found an association with maternal *TCF7L2* genotype.[14] Each maternal copy of the risk allele was associated with a 30 grams increase in offspring birth weight, probably as a result of higher maternal glucose levels stimulating fetal insulin production.[14]. After adjustment for maternal genotype, fetal *TCF7L2* genotype did not influence fetal birth weight.[14] This finding was replicated in the Helsinki birth cohort.[15] In another study, no association was found between fetal *TCF7L2* genotype and the risk of small size for gestational age.[16] Birth weight might be an inappropriate measure of the individual growth potential since different fetal growth rates may lead to the same birth weight.[17] Furthermore, rapid postnatal weight gain, especially in fat mass, has also been shown to be associated an increase risk of obesity and type 2 diabetes in later life, independent of birth weight.[18,19]

Therefore we hypothesized that longitudinally measured fetal and postnatal growth are better parameters in the investigation of the possible effect of *TCF7L2* on growth than specific growth characteristic such as birth weight. We first assessed the associations of *TCF7L2* rs7903146 with fetal and postnatal growth characteristics in a population-based prospective cohort study among 3,419 subjects followed from early fetal life onwards. Second, we assessed associations of this genotype with birth weight and postnatal

growth in 566 small-for-gestational-age (SGA) children participating in an independent cohort study.

Methods

Cohort Descriptions

The Generation R Study

The Generation R Study is a population based prospective cohort study from early fetal life onwards. The study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood. It has been described previously in detail.[20,21] Fetal and postnatal growth and their main determinants were repeatedly measured by physical examinations, fetal ultrasounds, biological samples and questionnaires. We have previously shown that of all eligible children born in the study area 61% participated in the study.[21] The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants or their parents.

Fetal growth and birth characteristics

Fetal ultrasound examinations were carried out during visits to one of the research centers. These fetal ultrasounds were used for establishing gestational age in the first trimester of pregnancy (conception to 12 weeks of gestational age), as well as for assessing fetal growth characteristics in second (17-25 weeks of gestational age) and third trimesters (>25 weeks of gestational age) of pregnancy.[22] Fetal growth measurements used in the present study included head circumference (HC), abdominal circumference (AC) and femur length (FL) measured in second and third trimesters to the nearest mm using standardized ultrasound procedures.[23] Estimated fetal weight (EFW) was calculated by means of the formula from Hadlock using head circumference, abdominal circumference and femur length ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 $(HC)^2$ – 0.003685 (AC * FL)).[24] First trimester ultrasound measures were not included for assessing growth characteristics because these ultrasound examinations were primarily performed to establish gestational age.

Birth and postnatal growth

Birth weight, date of birth and gender were obtained from community midwife and hospital registries. Information on head circumference or length at birth was not available, but many children were measured during the first two months of life. Well-trained staff in community health centers obtained postnatal growth characteristics using standard-

ized procedures. Based on the routine health care program, the visits at which these growth characteristics were measured were grouped into three age periods: 6 months (range 5 to 8.99); 12 months (range 9 to 12.99); and 24 months (range 23 to 34.99 months). Postnatally, head circumference was not measured at the age of 24 months.

Population for analysis

Analyses were restricted to singletons from whom DNA was available for *TCF7L2* genotyping and who also had Dutch or other Caucasian ethnicity as defined by having both parents born in the Netherlands or another European country (n = 3,419). Fetal growth measurements were available for 3,320 and 3,384 children in second and third trimesters, respectively. Of these children, those living outside the study area postnatally (10%) were not followed up in infancy and a further 12% were lost during postnatal follow-up, leaving 2,675 subjects eligible for the postnatal analyses.

The SGA Cohort

The SGA cohort was designed for the purpose of assessing growth and development of subjects born SGA. Subjects were included at childhood age (n = 367) or at young adult age (n = 252). Children were included in the SGA cohort when they were SGA at birth, had short stature (height standard deviation score (SDS) for age and gender of below – 2),[25] did not show catch-up growth in height, and had no growth failure caused by any other identified disorder. These inclusion criteria have previously been described.[26] Young adults included in the SGA cohort were randomly selected from hospitals in the Netherlands, where they had been registered because of being SGA. Only those young adults born at 36 weeks or more of gestation, being singleton and Caucasian and not suffering from conditions or receiving treatment known to interfere with growth, were invited to participate. SGA was defined as a birth length and/ or birth weight SDS of below –2.0 for gestational age.[27] The Medical Ethics Committees of Erasmus Medical Center, Rotterdam, and of the participating centers approved all studies and written informed consent was obtained from all participants or their parents.

Birth and postnatal growth

Birth characteristics of the SGA cohort were collected from hospital registries. The gestational age of the subjects was determined by ultrasound in the first trimester, if available, and otherwise calculated from the date of the last menstruation. Growth data (head circumference, height and weight) measured during the first two years of life were collected from records of hospitals, community health services and general practitioners. Longitudinal growth data were available in 272 participants in the SGA cohort .

Genotyping

DNA was collected from cord blood samples in the Generation R cohort and from peripheral venous blood samples in the SGA cohort. Cord blood for DNA isolation was available for 59% of all participating children of the Generation R cohort. When cord blood samples were missing, this result was mainly due to logistical constraints at the delivery. Venous blood samples were available in the complete SGA cohort. Genotyping of the C>T substitution in *TCF7L2* (rs7903146) gene was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 minutes), with 40 cycles of 94° C (15 seconds) and 60° C (1 minute)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 98% and 91% of the samples in the Generation R and SGA cohort, respectively. To confirm the accuracy of the genotyping results, 276 randomly selected samples from the Generation R Study were genotyped for a second time with the same method. The error rate was less than 1%. The frequency distribution in Generation R did not deviate from the Hardy-Weinberg equilibrium in subjects with Dutch ethnicity nor did it deviate in the SGA cohort.

Data analysis

With sample sizes in the Generation R Study of 3,419 and 2,675 subjects for fetal and postnatal analyses respectively, and assuming a statistical power level ($1 - \beta$) of 0.80, a level of significance (α) of 0.05 and a variance of 1.0, we were able to detect differences in growth characteristics of 0.048 SDS and 0.054 SDS respectively. First, differences in allele distribution between children born SGA (from the SGA cohort) and non-SGA subjects (from Generation R) were assessed. Differences were calculated using the Chi-square test. Second, we examined the differences in birth characteristics between genotype groups with linear regression analyses assuming an additive model. Weight, length and head circumference at birth and at different ages were analyzed using gender and age adjusted standard deviation scores (SDS).[27,28] Standard deviation scores were obtained using Dutch reference growth curves (Growth Analyser 3.0, Dutch Growth Research Foundation). For Generation R, we used the first length SDS and head circumference SDS measured after birth and before the second month of life, since these measurements were not available at birth. Third, we compared fetal (only Generation R) and postnatal characteristics between the genotypes with linear regression analyses. Finally, to assess longitudinally measured weight and length patterns from fetal life to infancy, we performed repeated measures regression analysis in both cohorts with weight and

length from birth to 24 months as outcome variables. This regression technique takes the correlation of multiple measurements within one subject into account, assesses both the time-independent and time-dependent effect of *TCF7L2* genotype on growth, and allows for incomplete outcome data.[29] In these models, genotype was included as both intercept and interaction with age. To account for (gestational) age at each specific measurement, these analyses were conducted with age-adjusted standard deviation scores. The models can be written as:

Height (SDS) or weight (SDS) = β_0 + β_1*age + β_2* *TCF7L2* genotype + β_3* *TCF7L2* genotype*age.

In this model, the term including 'β_0' reflects the intercept and the term including 'β_1' reflects the slope of growth (weight or length) per week for the reference group (CC genotype). The terms including 'β_2' and 'β_3' reflect the age independent growth differences in weight (and length) between the different categories of the *TCF7L2* genotype respectively.[30] All models were unadjusted (all growth characteristics are age and gender adjusted SD scores) since population genotype distribution is assumed to be unrelated to covariates and the effect estimates were not materially affected by adjusting for maternal age, pre-pregnancy body mass index or parity.[31] The occurrence of gestational diabetes in the entire cohort was 0.6% and did not affect the effect estimates. Therefore, occurrence of gestational diabetes was not included in the analyses.

All effect estimates are presented with their 95% confidence interval (95% CI). Statistical analyses were performed using the Statistical Analysis System version 9.1.3 (SAS, Stata corporation, College Station, TX, USA), including the PROC MIXED module for unbalanced repeated measurements as well as the Statistical Package of Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics of Generation R and SGA cohort are presented in Table 1. The minor allele frequency distributions did not differ significantly (p=0.47) between non-SGA subjects (from Generation R) (T-allele: 28.7%) and the SGA cohort (T-allele: 29.8%) (Table 2).

No significant differences between genotype groups were observed in fetal growth characteristics in Generation R (Table 3). No differences in birth characteristics (head circumference, length and weight), between genotype groups were observed in either cohort (Table 4). Postnatal growth characteristics for both cohorts are shown in Table 5.

Table 1: Subject characteristics by cohort.

Characteristics	Generation R	The SGA cohort
Gender (% boys)	50.8%	47.2%
Gestational age (weeks)	40.1 (36.7 – 42.4)	38.0 (29.9 – 41.0)
Birth weight (grams)	3513 (511)	1819 (716)
Premature (gestational age < 37 weeks) (%)	2.9%	44.9%
Birth weight < 2500 grams (%)	2.5%	82.7%
Small for gestational age (weight < -2 SDS) (%)	0.9%	100%
Gestational diabetes (%)	0.6%	N/A

Values are means (SD), medians (95% range) or percentages. N/A = not available

Table 2: Distribution of *TCF7L2* rs7903146 minor allele frequency according to cohort.

	Allele frequency		
	C-Allele	T-Allele	p-value
Non-SGA (Generation R) (%)	4828 (71.3)	1948 (28.7)	
SGA (SGA cohort) (%)	795 (70.2)	337 (29.8)	0.49

Non-SGA = All subjects from Generation R, excluding SGA subjects
SGA = birth weight SDS and/or birth length SDS < -2
p-value express differences in distribution between SGA and General population tested with Chi-square test.

Table 3: Fetal characteristics according to fetal *TCF7L2* rs7903146 genotype in the Generation R study.

	CC (n = 1736)	CT (n = 1329)	TT (n = 301)	p-value[#]
Fetal characteristics second trimester				
Head circumference (SDS)	0.04 (1.0)	0.02 (1.0)	0.05 (0.9)	0.88
Femur length (SDS)	-0.01 (1.0)	-0.01 (1.0)	0.05 (0.9)	0.64
Estimated fetal weight (SDS)	-0.06 (1.0)	-0.07 (1.0)	0.00 (1.0)	0.57
Fetal characteristics third trimester				
Head circumference (SDS)	0.11 (1.0)	0.13 (1.0)	0.15 (0.9)	0.45
Femur length (SDS)	0.01 (1.0)	-0.02 (1.0)	-0.04 (1.0)	0.34
Estimated fetal weight (SDS)	0.12 (1.0)	0.14 (1.0)	0.11 (0.9)	0.99

Values are means (SD). SDS = standard deviation score for age and gender.
[#] p-values for additive models. Differences were tested using linear regression analyses

No significant differences were found in either cohort for head circumference, weight or height at any age.

Finally, no differences were found in weight growth rate (SDS/year) from birth until the age of 2 years in either Generation R or the SGA cohort. Compared to the CC genotype, differences were -0.014 (95% confidence interval (CI): -0.064, 0.036) SDS/year and -0.028 (95% CI: -0.057, 0.002) SDS/year, for the CT and TT genotype, respectively, in Generation R. In the SGA cohort, differences were -0.134 (95% CI: -0.376, 0.108) SDS/year and 0.002 (95% CI: -0.125, 0.129) SDS/year, for the CT and TT genotype, respectively, using the CC

Table 4: Birth characteristics in both cohorts according to *TCF7L2* rs7903146 genotype of child.

Generation R		**CC (n = 1762)**	**CT (n = 1351)**	**TT (n = 306)**	**p-value#**
Gestational age (weeks)	n = 3419	40.3 (36.7 – 42.3)	40.3 (36.6 – 42.4)	40.1 (37.1 – 42.6)	0.83
Birth head circumference (SDS)*	n = 2314	0.22 (0.9)	0.24 (0.9)	0.26 (0.9)	0.55
Birth length (SDS)*	n = 1959	-0.07 (1.0)	-0.08 (1.0)	0.00 (1.1)	0.66
Birth weight (SDS)	n = 3419	0.21 (1.0)	0.22 (1.0)	0.20 (1.0)	0.97
SGA Cohort		**CC (n = 270)**	**CT (n = 255)**	**TT (n = 41)**	**p-value#**
Gestational age (weeks)	n = 566	38.0 (28.6 – 42.0)	38.0 (28.6 – 41.0)	38.0 (29.0 – 42.0)	0.57
Birth head circumference (SDS)	n = 203	-1.51 (1.4)	-1.20 (1.6)	-1.31 (1.4)	0.32
Birth length (SDS)	n = 491	-3.11 (1.4)	-3.27 (1.5)	-3.02 (1.5)	0.41
Birth weight (SDS)	n = 566	-2.40 (1.0)	-2.46 (0.9)	-2.32 (0.9)	0.58

** Length and head circumference were measured in the first two months of after birth.*
Values are means (SD) or medians (95% range). SDS = standard deviation score for age and gender.
 # p-values for additive models. Differences were tested using linear regression analyses.

Table 5: Postnatal characteristics at 6, 12, and 24 months according to *TCF7L2* rs7903146 genotype.

Generation R		**CC (n = 1375)**	**CT (n = 1063)**	**TT (n = 237)**	**p-value#**
6 months	Head circumference (SDS)	-0.02 (0.93)	-0.03 (0.89)	-0.06 (0.91)	0.83
n = 2675	Height (SDS)	0.03 (0.91)	0.03 (0.90)	0.07 (0.93)	0.81
	Weight (SDS)	0.41 (0.96)	0.44 (0.95)	0.54 (0.99)	0.14
12 months	Head circumference (SDS)	0.00 (0.89)	-0.04 (0.94)	-0.03 (1.12)	0.54
n = 2559	Height (SDS)	-0.01 (0.90)	-0.05 (0.90)	-0.01 (0.90)	0.70
	Weight (SDS)	0.18 (0.98)	0.18 (0.99)	0.24 (1.00)	0.63
24 months	Height (SDS)	-0.19 (0.93)	-0.21 (0.89)	-0.18 (0.87)	0.82
n = 2445	Weight (SDS)	-0.11 (0.99)	-0.13 (1.00)	-0.09 (0.96)	0.87
SGA cohort		**CC (n = 143)**	**CT (n = 107)**	**TT (n = 22)**	
6 months	Head circumference (SDS)	-1.38 (0.92)	-1.36 (0.90)	-1.74 (1.04)	0.41
n = 272	Height (SDS)	-2.39 (1.37)	-2.43 (1.26)	-2.51 (1.55)	0.93
	Weight (SDS)	-2.18 (1.40)	-2.22 (1.26)	-2.37 (2.05)	0.86
12 months	Head circumference (SDS)	-1.21 (0.83)	-1.24 (0.88)	-1.72 (1.06)	0.16
n = 268	Height (SDS)	-2.25 (1.25)	-2.30 (1.06)	-2.30 (1.47)	0.94
	Weight (SDS)	-2.14 (1.41)	-2.25 (1.15)	-2.17 (1.89)	0.82
24 months	Head circumference (SDS)	-1.10 (0.82)	-1.13 (0.87)	-1.60 (1.06)	0.20
n = 244	Height (SDS)	-2.39 (1.24)	-2.47 (1.05)	-2.94 (1.04)	0.20
	Weight (SDS)	-2.19 (1.33)	-2.31 (1.21)	-3.06 (1.65)	0.04

Values expressed as mean (SD). SDS = standard deviation score for age and gender.
 # p-values for additive models. Differences were tested using linear regression.

genotype as a reference. Similarly, no differences were found in height growth rate from birth to 2 years in either cohort (data not shown).

Discussion

In the current study, we found that T2D gene polymorphism TCF7L2 rs7903146 is not associated with growth in fetal life in the general population or with growth in early postnatal life in either the general population or in a cohort of subjects born SGA. We also confirmed previous suggestions that this variant of TCF7L2 is not associated with birth weight and, more importantly, demonstrated that it does not influence the fetal development using direct fetal measurements. Finally, we showed that this polymorphism does not appear to be associated with the risk of being born SGA.

To our knowledge, this study is the first to examine the association of *TCF7L2* with longitudinally measured growth patterns in fetal and early postnatal life in two independent birth cohorts. In the Generation R Study, DNA for genotyping was available in 59% of all subjects and was isolated from cord-blood. Missing cord-blood was mainly caused by logistical restraints at delivery. Children who were not genotyped had a shorter gestational age ($p < 0.001$) and were lighter at birth ($p < 0.001$) than subjects who were genotyped. Of all genotyped eligible subjects at baseline, 22% did not participate in follow-up measurements. In the SGA cohort, genotyping was successful in 91% of the subjects and longitudinally growth data were available in 48% of the cohort. Our effect estimates could be biased if the associations between genotypes and growth characteristics differed between those with and without postnatal growth data available. In the Generation R cohort, no differences were observed between children with and without postnatal growth measurements. In the SGA cohort the T-allele was slightly more frequent in subjects with postnatal growth measurements than in subjects without these measurements ($p < 0.05$). Finally, it could be possible that there is differential effect of genotype on growth according to availability of follow-up data. This bias would affect our estimates, though such a bias seems unlikely.

Several studies have investigated the effect of common genetic variants related to insulin action and secretion on early growth.[14,15,32,33] Of the initially identified T2D gene polymorphisms identified by the GWA, fetal *CDKAL1* (rs7754840) and *HHEX* (rs1111875) genotype, and maternal *TCF7L2* (rs7903146) genotype have been shown to affect birth weight. Pulizzi *et al*. demonstrated in the Helsinki Birth Cohort that fetal *TCF7L2* genotype did not interact with birth weight to increase the risk of T2D in adulthood.[15] *TCF7L2* rs7903146 has been shown to have the strongest genetic effect on T2D and this result has been replicated in several studies.[3-5] Therefore, *TCF7L2* is a very important candidate gene for explaining the association between low birth weight and T2D risk. Our study is

the first to investigate the effect of *TCF7L2* rs7903146 on longitudinal growth in early life. Longitudinal assessment of growth provides more information than just measurements at birth as we have demonstrated earlier that different fetal growth patterns may result in a similar birth weight.[17] Furthermore, most SGA born children have catch-up growth during the first months of life but 15% remain small.[34] Thus, to investigate whether *TCF7L2* rs7903146 influences fetal and postnatal growth, longitudinal growth data provide more information than birth weight alone.

Freathy *et al*. found an increase of birth weight for each fetal and maternal risk allele.[14] They concluded that the most likely mechanism for this association was that maternal genotype was associated with a reduction of maternal insulin secretion, leading to increased fetal glucose and insulin levels and subsequently increased birth weight, rather than a direct effect of the fetal genotype on birth weight. Pulizzi *et al*. found no effect of the fetal genotype of this polymorphism on birth weight. Since fetal and maternal genotypes are 50% correlated, it cannot be excluded that, when the risk allele is present in both mother and child, small effects of fetal genotype that reduce fetal growth could be masked by opposing effects of maternal genotype. Since maternal genotype was not available in our study, we were not able to test this hypothesis. However, we did not find any effect of fetal genotype on birth weight in the general population nor in a specific population of children with insufficient fetal growth resulting in small size for gestational age at birth. Our findings are therefore in line with the conclusions of these previous studies. Furthermore, we found no effect of fetal genotype on estimated fetal weight or weight during infancy, indicating that there is no evidence for any association between this fetal genotype and weight or change in weight during early life either. The effect of this polymorphism on the metabolic phenotype found in adults would therefore appear to develop after early childhood. Nonetheless, our results also could be explained by a lack of power and we cannot rule out that we were unable to detect smaller effects of this variant on early growth.

Regarding intra-uterine growth retardation, an earlier study examined the effect of *TCF7L2* rs7903146 genotype on SGA. Cauchi *et al*. found no association between this genotype and SGA, using family-based association analyses in over 3,000 subjects of which 627 subjects were SGA.[16] In this analyses, the SGA group was slightly larger than in our current study and included parents, but postnatal growth data were not analyzed longitudinally. In our study, we did not find a difference in minor allele frequency between the general population (Generation R) and the SGA cohort. On the basis of two independent and negative studies, one may conclude that there is no association between this genetic polymorphism and risk of SGA.

In summary, our results suggest that *TCF7L2* rs7903146 does not influence growth from early fetal life to infancy. Furthermore, minor allele frequency was not different in SGA subjects than in non-SGA subjects, indicating that it is unlikely that this polymor-

phism is associated with the risk of being born SGA. Systematic searches for common genetic variants by means of genome-wide association studies will enable us to obtain a more complete understanding of which genes are involved in growth in fetal life and infancy.

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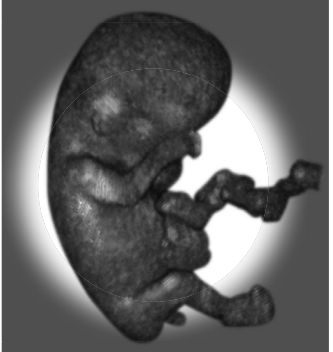

CHAPTER 3.4

PPARγ-2 polymorphism Pro12Ala, breastfeeding and growth in early life

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Abstract

Background: We examined whether the *PPARγ2* Ala12 allele influences growth in early life and whether this association is modified by breastfeeding.

Methods: This study was embedded in the Generation R Study, a prospective cohort study from early fetal life onwards. *PPARγ2* was genotyped in DNA obtained from cord-blood samples in 3,432 children. Information about breastfeeding was available from questionnaires. Weight, head circumference and (femur) length were repeatedly measured in second and third trimesters of pregnancy, at birth and at the ages of 1.5, 6, 11, 14 and 18 months.

Results: Genotype frequency distribution was 77.6% (Pro12Pro), 20.7% (Pro12Ala), and 1.7% (Ala12Ala). Growth rates in weight from second trimester of pregnancy to 18 months were higher amongst the Pro12Ala and Ala12Ala compared to Pro12Pro carriers (differences: 1.11 (95% confidence interval (CI): 0.47, 1.74) and 2.65 (95%CI: 0.45, 4.87) grams/week, respectively). We found an interaction between genotype and breastfeeding duration (p-value interaction <0.0001). In infants who were breastfed for at least four months, *PPARγ2* Pro12Ala was not associated with growth rate. When breastfeeding duration was shorter than two months or between two and four months, growth rate was higher in Ala12Ala subjects than Pro12Pro (differences: 9.80 (95%CI: 3.97, 15.63) and 6.32 (95%CI: -1.04, 13.68) grams/week, respectively).

Conclusions: The *PPARγ2* Ala12 allele is associated with an increased growth rate in early life. This effect may be influenced by breastfeeding duration. Further studies should replicate these findings, identify the underlying mechanisms, and assess whether these effects persist into later life.

Introduction

Previous studies have shown that common polymorphisms of peroxisome proliferator-activated receptor γ-2 (*PPARγ2*) are associated with adipocyte differentiation, lipid metabolism and insulin sensitivity.[1] Recent genome-wide association (GWA) studies found consistent and robust associations of the PPARγ2 Pro12Ala polymorphism (rs1801282) with type 2 diabetes.[2,3] Furthermore, several studies have reported an increase in body mass index (BMI) in *PPARγ2* Ala12 carriers.[4-6] In a meta-analysis, Masud et al. found in adults with a BMI of more than 27 kg/m2 that carriers of the *PPARγ2* Ala12 allele had an increase in BMI. Also, they found a significant increase in BMI in Ala12Ala carriers in comparison with Pro12Pro carriers in subjects with a normal BMI. However, a recent GWA study in more than 80,000 subjects on BMI did not identify the *PPARγ2* Ala12 allele as a variant associated with BMI in the general adult population.[7] Among children, it has been suggested that *PPARγ2* Ala12 allele is associated with BMI, though the evidence is very limited. In a small study, carriers of the *PPARγ2* Ala12 allele have been shown to be heavier at the age of 7 years,[5] and in a cohort of children at the age of 1 to 6 years, an association of *PPARγ2* Ala12 allele with increased adiposity was only found in girls aged 3 to 4 years.[6]

Common polymorphisms of PPARγ2 may also explain previously suggested associations of growth in early fetal life and infancy with obesity.[8] This association may be explained by early, modest alterations in insulin secretion and sensitivity since insulin is the most important fetal growth hormone.[9] The effect of the Ala12 allele on anthropometrics and growth patterns may already be present in fetal life and infancy. In two large studies, no association was found between *PPARγ2* and birth weight, though an association with preterm birth has been suggested.[10-12] Birth weight alone might be an inappropriate measure of the individual growth potential since different fetal growth rates may lead to the same birth weight.[13] Moreover, most growth-restricted infants catch up to their own genetically determined growth curve during the first years postnatally.[14] The *PPARγ2* Ala12 allele has been found to interact with birth weight in determining further growth patterns.[15] Also, the effect of *PPARγ2* genotype on metabolic phenotype appears to depend on dietary intake.[16-18] No previous studies have examined the effect of breastfeeding on the association of *PPARγ2* genotype and growth in early life, while breastfeeding is well known to influence early growth and has a protective effect on the risk of obesity in childhood.[19-21]

Based on previous findings, we hypothesized that the *PPARγ2* Ala12 allele is associated with increased weight gain during early life and that this association might be influenced by breastfeeding duration. We examined in a large prospective birth cohort study from fetal life onwards the association of the Pro12Ala polymorphism in the *PPARγ2* gene

with growth in fetal life and infancy and whether this association may be modified by breastfeeding.

Methods

Design

This study was embedded in the Generation R Study, a prospective cohort study from early fetal life onwards. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail.[22,23] Fetal and postnatal growth and their main determinants were repeatedly measured by physical examinations, fetal ultrasounds, biological samples and questionnaires. We have previously shown that of all eligible children born in the study area 61% participated in the study.[22] The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all parents.

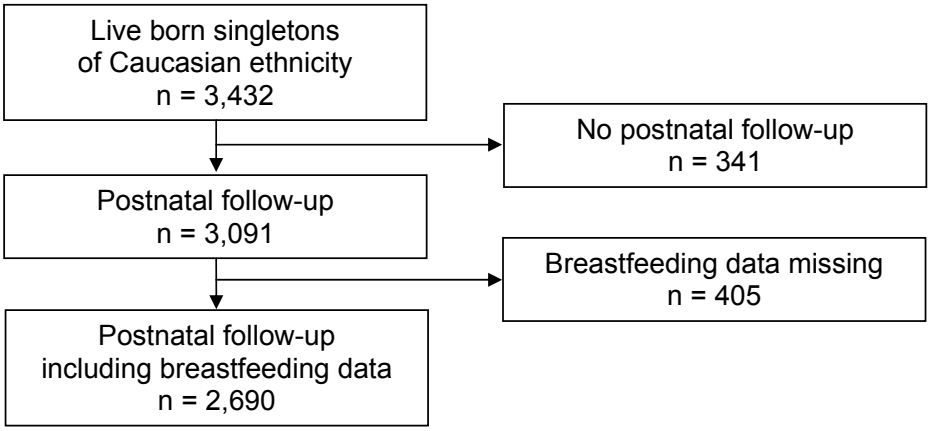
Population for analysis

Analyses were restricted to children from whom DNA was available for *PPARγ2* genotyping and with Dutch or other European Caucasian ethnicity as defined by having both parents born in the Netherlands or another European country (n = 3,432). Fetal growth measurements were available in 3,331 and 3,398 children in second and third trimester, respectively. Of these children, those living outside the study area postnatally (n = 341) were not followed up in infancy, leading to 3,091 subjects for the postnatal growth measurements (Figure 1). Of all children followed postnatally, data on breastfeeding were missing in 405 subjects, leaving 2,690 children with complete data on postnatal growth and breastfeeding duration. Of all genotyped subjects at baseline, the mean follow-up rate per visit was 77%.

Genotyping

DNA was collected from cord blood samples at birth. Cord blood for DNA isolation was available in 59% of all live-born participating children. Missing cord blood samples were mainly due to logistical constraints at the delivery. Genotyping of the *PPARγ2* gene Pro12Ala polymorphism (rs1801282) was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg, Germany). The genotyping reaction was amplified using the GeneAmp® PCR

Figure 1: Flow diagram indicating number of subjects for each analysis.



system 9600 (95° C (15 minutes), then 40 cycles of 94° C (15 seconds) and 60° C (1 minute)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 99% of the samples. To confirm the accuracy of the genotyping results 276 randomly selected samples were genotyped for a second time with the same method. The error rate was 0%.

The genotype distribution (Pro12Pro 77.6%, Pro12Ala 20.7%, Ala12Ala 1.7%; minor allele frequency (Ala) of 22.4%) was similar to those found in previous studies and the frequency distribution did not deviate from the Hardy-Weinberg equilibrium (chi-square = 1.82, p=0.18).[4]

Fetal growth and birth characteristics

Fetal ultrasound examinations were carried out during visits to one of the research centers. These fetal ultrasounds were used for establishing gestational age in the first trimester and for assessing fetal growth characteristics in second and third trimesters of pregnancy.[24] Fetal growth characteristics measurements used for the present study included head circumference (HC), abdominal circumference (AC) and femur length (FL) measured in second and third trimesters to the nearest mm using standardized ultrasound procedures.[25] Estimated fetal weight (EFW) was calculated using the formula by Hadlock using head circumference, abdominal circumference and femur length ($\log_{10}$ EFW = 1.5662 – 0.0108 (HC) + 0.0468 (AC) + 0.171 (FL) + 0.00034 $(HC)^2$ – 0.003685 (AC * FL)).[26] First trimester ultrasound measures were not included as growth characteristics since these ultrasound examinations were primarily performed to establish gestational age.

Postnatal growth

Birth weight, date of birth and gender were obtained from community midwife and hospital registries. Information on head circumference or length at birth was not available. Well-trained staff in community health centers obtained postnatal growth characteristics using standardized procedures. Weight was measured using electronic scales (SECA, Hamburg, Germany). Length was determined in supine position to the nearest millimeter until the age of 6 months using a neonatometer, after which it was measured in upright position (Holtain Limited, Dyfed, United Kingdom). Head circumference was measured to the nearest millimeter using a standardized tape (SECA, Hamburg, Germany). Based on the routine health care program, visits for measurements of these growth characteristics were grouped into five age periods: 1.5 (range 0 to 3.99) months; 6 (range 4 to 9.99) months; 11 (range 10 to 12.99) months; 14 (range 13 to 16.99) months; and 18 (range 17 to 20.99) months. Postnatally, head circumference was only measured at 1.5, 6 and 14 months.

Breastfeeding

Information about duration of breastfeeding was obtained by postnatal questionnaires at the ages of 2, 6 and 12 months. This information was combined to form the following categories: (1) breastfed 0 to 2 months, (2) breastfed 2 to 4 months and (3) breastfed longer than 4 months.

Covariates

Information on maternal age, educational level, parity and weight before pregnancy was obtained by the first questionnaire at the enrolment in the study. Maternal height was measured without shoes at our research center, and body mass index (weight/height^2 (kg/m^2)) was calculated. The occurrence of gestational diabetes was obtained from midwife or obstetric records.

Data analysis

We explored the differences in gestational age (weeks) and birth weight (SDS) between the three genotypes with additive, dominant (*PPARγ2* Ala12Ala/Pro12Ala vs. Pro12Pro) and recessive (*PPARγ2* Pro12Pro/Pro12Ala vs. Ala12Ala) models using Mann-Whitney U-tests and linear regression. To assess longitudinally measured growth characteristics from fetal life to infancy, we performed unbalanced repeated measures regression analysis with weight, length and head circumference in fetal life and infancy as outcomes.

This regression technique takes the correlation of multiple measurements within one subject into account, assesses both the time-independent and time-dependent effect of *PPARγ2* Pro12Ala genotype on growth, and allows for incomplete outcome data.[27] The best-fitting model as a function of (gestational) age was constructed using fractional polynomials.[28] To account for differences in growth curves for weight, length and head circumference in fetal life and infancy, growth models were constructed for three age periods: second trimester to 18 months (overall), second trimester to birth (fetal), and birth to 18 months (infancy). In the 'fetal' and 'overall' models, age was defined as age in weeks after conception. For the 'infancy' model, age represented biological age in weeks and these models were additionally adjusted for gestational age at birth. In all models, genotype was included as both intercept and interaction with age to take account of differences at baseline and in growth rates. The models used are shown in the Appendix. Finally, we examined the interaction between genotype and breastfeeding duration and the effect on growth rate. Trend tests were performed by adding both genotype and breastfeeding duration in the model as ordinal variables instead of a categorical variables. All models were adjusted only for gender since population genotype distribution is assumed to be unrelated to covariates, and the effect estimates were not materially affected by adjusting for covariates such as maternal age, educational level, pre-pregnancy body mass index or parity.[29] The occurrence of gestational diabetes in the entire cohort was 0.6% and did not differ between the genotypes and was therefore not included in the analyses. All effect estimates are presented with their 95% confidence interval (95% CI). Statistical analyses were performed using the Statistical Analysis System, Version 8.2 (SAS, Stata Corporation, College Station, TX, USA), including the PROC MIXED module for unbalanced repeated measurements and the Statistical Package of Social Sciences, Version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Tables 1 and 2 show the subjects characteristics. In the whole study group, mean birth weight was 3,512 (standard deviation (SDS): 510) grams, median gestational age 40.3 (95% range 36.7 – 42.4) weeks. The percentages of children born prematurely and with low birth weight were 2.9% and 2.4%, respectively. The median number of measurements (visits) per subject was 7 (95% range: 3-8 visits). Table 3 shows the differences in birth characteristics between the *PPARγ2* Pro12Ala genotypes. No significant differences were found in gestational age and birth weight (SDS). Pro12Ala and Ala12Ala carriers tended to have an increased risk of preterm birth, though these differences were not significant (Odds Ratios, using the Pro12Pro genotype as a reference: 1.51 (95% confidence interval (CI): 0.96, 2.38) for Pro12Ala, and 2.08 (95% CI: 0.64, 6.80) for Ala12Ala).

Table 1: Maternal and birth characteristics of 3,432 children.

Maternal Characteristics	
Age (years)	30.8 (4.8)
Weight before pregnancy (kg)	66.9 (11.8)
Height (cm)	170 (6.7)
Body mass index before pregnancy (kg/m2)	22.9 (3.7)
Parity (% nulliparous)	60.1%
Gestational diabetes (%)	0.6%
Placental weight (grams)	640 (142)
Educational level (%)	
Primary school	4.5%
Secondary school	39.1%
Higher education	56.5%
Birth characteristics	
Gender (% boys)	50.8%
Gestational age (weeks)	40.3 (36.7 – 42.4)
Birth weight (grams)	3512 (510)
Premature (gestational age < 37 weeks) (%)	2.9%
Birth weight < 2500 grams (%)	2.4%
Small for gestational age (< -2 SDS) (%)	2.4%
Breastfeeding duration (%)	
Between 0 and 2 months	31.3%
Between 2 and 4 months	26.4%
Longer than 4 months	42.3%
Number of visits	
Total	7 (3 – 8)

Values are means (SDS), medians (95% range) or percentages. Of the total group, data were missing on weight before pregnancy (n=468), height (n=5), body mass index before pregnancy (n=470), parity (n=3), gestational diabetes (n=39), placental weight (n=792), educational level (n=45) and breastfeeding duration (n=742).

Table 4 shows the differences in growth rates between the *PPARγ2* genotypes using Pro12Pro as the reference group. In fetal life, *PPARγ2* Ala12Ala carriers tended to show an increased growth rate in weight compared to the Pro12Pro carriers. Postnatally, both the Pro12Ala and Ala12Ala carriers have an increased growth rate in weight compared to Pro12Pro carriers, though this was not significant in the latter. Furthermore, we observed an allele dose effect for each additional Ala12 allele (p=0.0092). For weight gain over the entire period from second trimester to 18 months, *PPARγ2* Pro12Pro carriers had a significantly lower growth rate for weight than the other genotypes and there was a significant trend for each additional Ala12 allele (p<0.0001). Figure 2 shows the estimated weight differences in grams between the genotype groups. The estimated

Table 2: Fetal and postnatal growth characteristics of 3,432 children.

Second trimester	
Gestational age at visit (weeks)	20.5 (18.6 – 23.0)
Head circumference (cm)	18.0 (1.4)
Femur length (mm)	33.3 (3.4)
Estimated fetal weight (grams)	380 (87)
Third trimester	
Gestational age at visit (weeks)	30.4 (28.5 – 32.7)
Head circumference (cm)	28.6 (1.2)
Femur length (mm)	57.4 (2.8)
Estimated fetal weight (grams)	1628 (249)
Birth	
Gestational age (weeks)	40.3 (36.7 – 42.4)
Weight (grams)	3512 (510)
1.5 months	
Age at visit (months)	1.3 (0.9 – 3.0)
Head circumference (cm)	38.3 (1.5)
Length (cm)	55.8 (2.8)
Weight (grams)	4788 (728)
6 months	
Age at visit (months)	6.1 (4.5 – 7.7)
Head circumference (cm)	43.5 (1.3)
Length (cm)	67.7 (2.6)
Weight (grams)	7798 (862)
11 months	
Age at visit (months)	11.0 (10.1 – 12.5)
Length (cm)	74.4 (2.5)
Weight (grams)	9644 (999)
14 months	
Age at visit (months)	14.2 (13.5 – 15.7)
Head circumference (cm)	47.2 (1.3)
Length (cm)	78.4 (2.7)
Weight (grams)	10532 (1089)
18 months	
Age at visit (months)	18.3 (17.3 – 20.4)
Length (cm)	82.4 (3.0)
Weight (grams)	11556 (1214)

Values are means (SDS) or medians (95% range).

Table 3: Birth characteristics according to *PPARγ2* Pro12Ala genotypes in 3,432 children.

	Pro12Pro (n = 2664)	Pro12Ala (n = 710)	Ala12Ala (n = 58)	p-value Additive	p-value Dominant	p-value Recessive
Gestational age (weeks)	40.3 (36.9 – 42.4)	40.3 (36.4 – 42.4)	40.4 (32.3 – 42.9)	0.35	0.69	0.91
< 37 weeks (% (n))	2.6 (68)	3.8 (27)	5.2 (3)			
> 37 weeks (% (n))	97.4 (2596)	96.2 (683)	94.8 (55)			
Birth weight (SDS)	0.04 (0.98)	0.04 (1.03)	0.28 (0.97)	0.37	0.66	0.07
< -2 SDS (% (n))	2.2 (59)	3.4 (24)	0.0 (0)			
> -2 SDS (% (n))	97.8 (2605)	96.6 (686)	100.0 (58)			

Values represent means (SDS) or medians (95% range) for continuous variables and number of subjects for dichotomous variables. Differences were tested using Mann-Whitney U-test or linear regression. Dominant model: PPARγ2 Ala12Ala/Pro12Ala vs. Pro12Pro. Recessive model: PPARγ2 Pro12Pro/Pro12Ala vs. Ala12Ala.

differences at 18 months compared to the Pro12Pro group were 99 and 291 grams for the Pro12Ala and Ala12Ala carriers, respectively. Prenatal head circumference growth rate was significantly lower in Ala12Ala compared to Pro12Pro carriers. No other significant differences were found in growth rate of head circumference or length.

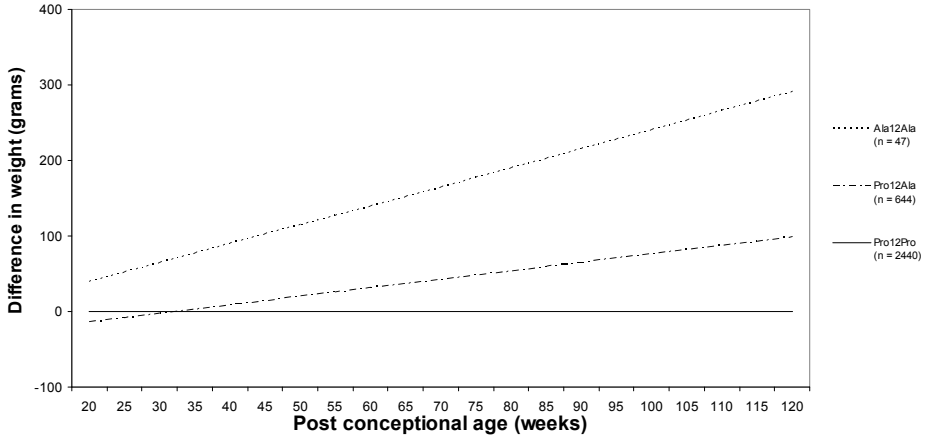
We found a significant interaction between genotype and breastfeeding duration on growth rate in weight (p for interaction <0.0001). Figure 3 shows the differences in estimated growth rates between the genotypes in the three breastfeeding groups. When breastfeeding duration was shorter than two months or between two and four months, growth rate was higher in Ala12Ala carriers than Pro12Pro (differences: 9.80 (95%CI: 3.97, 15.63) grams/week and 6.32 (95%CI: -1.04, 13.68) grams/week, respectively). No associations of *PPARγ2* with growth rate were found in children who were breastfed more than 4 months. Table 5 shows the trends within each genotype and breastfeeding group. The maximum difference in growth rate was found between *PPARγ2* Ala12Ala carriers who were breastfed shorter than two months and Pro12Pro carriers who were breastfed longer than four months (difference: 12.62 grams/week (95% CI: 6.80, 18.44) grams/week). Similar effects were found in subjects who were breastfed for 2 to 4 months and we found an allele dose effect for each additional Ala12 allele in these subjects (p=0.0264). All effect estimates did not materially change after restricting analyses to term born subjects (> 37 weeks of gestation) or after adjusting for covariates, such as maternal age, educational level, pre-pregnancy body mass index or parity (data not shown). No interactions between genotype and breastfeeding on length or head circumference growth rate were observed (data not shown).

Table 4: Differences in growth rate for weight by *PPARγ2* Pro12Ala genotypes using repeated measures regression analysis.

Genotype	Difference in weight gain from second trimester to birth (grams / week) (n=3,432)	Difference in weight gain from birth to 18 months (grams / week) (n=3,091)	Difference in weight gain from second trimester to 18 months (grams / week) (n=3,091)
Pro12Pro	Reference	Reference	Reference
Pro12Ala	0.50 (-0.96, 1.98) p = 0.4988	1.06 (0.02, 2.10) p = 0.0454	1.11 (0.47, 1.74) p = 0.0007
Ala12Ala	4.02 (-0.59, 8.63) p = 0.0876	3.52 (-0.05, 7.10) p = 0.0535	2.65 (0.45, 4.87) p = 0.0185
	p-value for trend = 0.1575	p-value for trend = 0.0092	p-value for trend < 0.0001
	Difference in head circumference gain from second trimester to 1.5 months (mm * 10^{-1} / week) (n=3,432)	**Difference in head circumference gain from 1.5 months to 14 months (mm * 10^{-1} / week) (n=3,091)**	**Difference in head circumference gain from second trimester to 14 months (mm * 10^{-1} / week) (n=3,091)**
Pro12Pro	Reference	Reference	Reference
Pro12Ala	-0.16 (-0.51, 0.18) p = 0.3617	0.01 (-0.01, 0.03) p = 0.1587	0.05 (-0.09, 0.19) p = 0.4725
Ala12Ala	-1.40 (-2.56, -0.23) p = 0.0186	0.02 (-0.05, 0.10) p = 0.5104	-0.48 (-1.01, 0.02) p = 0.0643
	p-value for trend = 0.0566	p-value for trend = 0.1293	p-value for trend = 0.8368
	Difference in length gain from second trimester to 1.5 months (mm * 10^{-1} / week) (n=N/A)	**Difference in length gain from 1.5 months to 18 months (mm * 10^{-1} / week) (n=3,091)**	**Difference in length gain from second trimester to 14 months (mm * 10^{-1} / week) (n=N/A)**
Pro12Pro	N/A	Reference	N/A
Pro12Ala	N/A	-0.11 (-0.37, 0.14) p = 0.3864	N/A
Ala12Ala	N/A	0.43 (-0.44, 1.31) p = 0.3299	N/A
		p-value for trend = 0.4429	

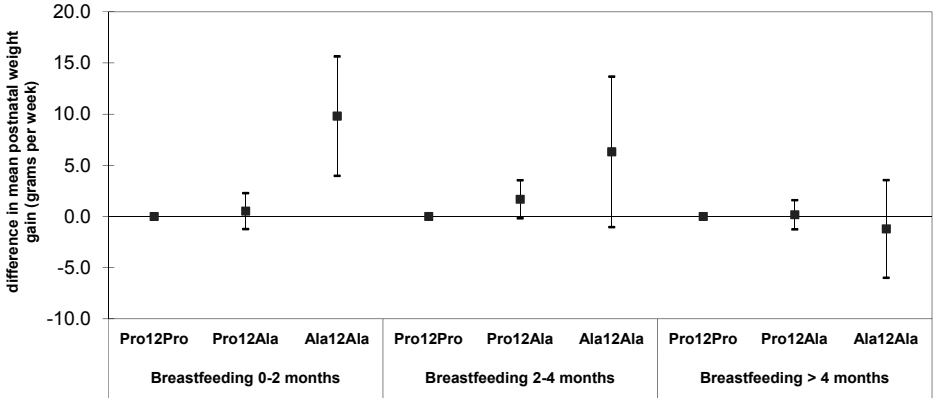
Values are regression coefficients (95% confidence interval) and reflect the difference in growth rate. Models are adjusted for gender of the child. Analyses focused on growth during infancy are additionally adjusted for gestational age at birth. Estimates based on repeated measures regression analysis. Total number of subjects is 3,432 for growth from second trimester to birth/1.5 months (Pro12Pro n=2664; Pro12Ala n=710; Ala12Ala n=58); total number of subjects is 3,091 for postnatal growth and growth over the entire period (Pro12Pro n=2440; Pro12Ala n=644; Ala12Ala n=47).

Figure 2: Differences in weight between fetal *PPARγ2* Pro12Ala genotypes using Pro12Pro as the reference in 3,091 children.



Values reflect the difference in weight (grams) and are based on repeated measures regression analysis, using the following model:
Weight (grams) $= \beta_0 + \beta_1 * age + \beta_2 * age^2 + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 * gender.$

Figure 3: Differences in postnatal weight between fetal *PPARγ2* Pro12Ala genotypes stratified by duration of breastfeeding in 2,690 children.



Values are regression coefficients (95% confidence interval) and reflect the difference in weight gain (grams/week). Model is adjusted for gestational age at birth and gender of the child. Estimates based on repeated measures regression analysis.

Discussion

In this study we showed that the Ala allele of the *PPARγ2* Pro12Ala gene polymorphism (rs1801282) is associated with an increased growth rate in early life. Between the second trimester of pregnancy and 18 months, children with Pro12Ala and Ala12Ala had higher

Table 5: Interaction between fetal *PPARγ2* Pro12Ala genotype and breastfeeding in relation to growth rate (grams/week) between birth and 18 months in 2,690 children.

Genotype	Breastfeeding duration			
	0-2 months (n=843)	2-4 months (n=710)	> 4 months (n=1137)	p-value for trend
Pro12Pro (n=2079)	2.82 (1.78, 3.85) n = 663, p < 0.0001	1.37 (0.28, 2.46) n = 547, p = 0.0135	0 (Reference) n = 869	< 0.0001
Pro12Ala (n=570)	3.34 (1.64, 5.05) n = 166, p = 0.0001	3.05 (1.27, 4.84) n = 155, p = 0.0008	0.17 (-1.26, 1.59) n = 249, p = 0.8170	0.0013
Ala12Ala (n=41)	12.62 (6.80, 18.44) n = 14, p < 0.0001	7.69 (0.35, 15.03) n = 8, p = 0.0399	-1.21 (-5.99, 3.56) n = 19, p = 0.6183	0.0003
p-value for trend	0.0432	0.0264	0.9555	interaction: < 0.0001

Values are regression coefficients (95% confidence interval) and reflect the difference in weight gain from birth to 18 months (grams / week). Model is adjusted for gestational age at birth and gender of the child. Estimates based on repeated measures regression analysis.

growth rates in weight than Pro12Pro subjects. Furthermore, our results suggest that the effect of *PPARγ2* gene polymorphism on growth in infancy depends on breastfeeding duration.

To our knowledge, this study is the first prospective cohort study that examined the association of this *PPARγ2* Pro12Ala gene polymorphism with growth from fetal life until infancy. DNA for genotyping was available in 59% of all subjects. Missing cord blood and DNA was mainly caused by logistical constraints at delivery. Children who were not genotyped had a shorter gestational age ($p < 0.001$) and were lighter at birth ($p < 0.001$) than subjects who were genotyped. Of all genotyped subjects at baseline, the mean follow-up rate per visit was 77%. Response rates were lowest at the age of 18 months. This is mainly due to general lower response rate at this age in the routine childcare system. In our data, no differences in genotype frequency or birth characteristics were observed between children with and without postnatal growth measurements. Furthermore, similar results were observed when analyses were restricted to children aged 11 or 14 months and when breastfeeding duration was dichotomized into shorter or longer than 3 months (data not shown). Our effect estimates would be biased if the associations between genotypes and growth characteristics differed between those with and without postnatal growth data available. This seems unlikely. Finally, similar to other studies, the number of *PPARγ2* Ala12Ala carriers was small, especially after stratifying for breastfeeding duration.[4] Despite the low numbers of subjects in this group, we found significant effects in Ala12Ala carriers. Furthermore, we found effects in Pro12Ala carriers and significant trend effects for each additional Ala12 allele.

Several studies have found associations between this *PPARγ2* Pro12Ala gene polymorphism and body composition.[4,30,31] A meta-analysis demonstrated that the Ala12Ala

is associated with a higher body mass index in adulthood.[4] However, in a recent very large GWA study, the *PPARγ2* Ala12 allele was not identified as a BMI variant in adulthood.[7] A number of studies have suggested that *PPARγ2* Ala12 allele may be associated with adiposity during childhood, though the evidence in limited.[5,6,15] In a small study, Pihlajamäki *et al*. showed that children with the Ala12 allele were heavier at the age of 7 years.[5] Also, in a cohort of children from 1 to 6 years, an association of the *PPARγ2* Ala12 allele with increased adiposity was only found in girls aged 3 to 4 years.[6] Two large birth cohort studies found no association between this *PPARγ2* polymorphism and birth weight.[10,11] Eriksson *et al*. described a 'gene-birth weight' interaction in adults, where individuals with the Ala12 allele and a lower birth weight were at risk of increased lipid levels.[32]

No other studies have examined the effect of this polymorphism on growth in fetal life and infancy in a large prospective cohort. Most studies on weight and body composition were performed retrospectively on cross-sectional data. We believe that if this *PPARγ2* polymorphism is truly associated with growth in early life, associations with longitudinally measured growth patterns might be expected to be stronger than with only one or two growth measurements. Our study showed that carriers of at least one *PPARγ2* Ala12 allele had an increased growth rate in weight until the age of 18 months. The postnatal effect on growth rate appeared to be dependent of duration of breastfeeding. No association was found between genotype and growth rate in children who had received at least four months of breastfeeding. Among children who were breastfed for less than four months, a significant positive effect on growth rate of up to almost 10 grams per week was found in the *PPARγ2* Ala12 allele carriers. The size of the effect was inversely related to breastfeeding duration. These findings may indicate a possible gene-nutrition interaction with regards to growth rate.

There have been a limited number of previous studies that also have suggested a gene-nutrition interaction concerning this *PPARγ2* polymorphism. In a large cohort, Memisoglu *et al*. found that the relationship between dietary fat intake and body mass index was dependent on *PPARγ2* genotype.[33] In this study, dietary fat intake was strongly associated with an increased risk of obesity among Pro12Pro carriers while no association was reported among carriers of the Ala12 allele. Luan *et al*. found that dietary fat intake (expressed as polyunsaturated-to-saturated fat ratio) was not associated with body mass index or fasting insulin levels in Pro12Pro carriers, but was inversely related in these outcomes in Ala12 allele carriers.[17] Other studies, however, were not able to replicate these results.[18,33] Our results would be in line with the findings of Luan *et al*. based on the assumption that breast milk contains more polyunsaturated fatty acids than formula.[34] Longer breastfeeding duration could lead to a higher polyunsaturated fat intake, and subsequently, to a relatively lower growth rate amongst Ala12Ala carriers who were breastfed compared to those who were formula-fed. Breastfeeding has also

been indicated to have a protective effect on the risk of obesity, though the effect appears to be limited.[19-21] Our results suggest that this protective effect might be modified by *PPARγ2*, since we found the highest growth rates in children who were never breastfed or breastfed for less than 4 months and were Ala12 allele carriers. Furthermore, rapid weight gain in the first months of life is associated with increased risk of obesity in childhood.[35] Based on the current study, it could be hypothesized that the interaction between *PPARγ2* and breastfeeding plays an important role in this association.

From the current data, however, it remains unclear whether breastfeeding reduces the risk of an increased growth rate in *PPARγ2* Ala12 allele carriers or that formula feeding increases that same risk. The associations may be explained by either growth stimulating or metabolism inhibiting activities among Ala12 allele carriers. A previous study has shown that the *PPARγ2* Ala12 allele is associated with a moderate reduction in *PPARγ2* transcriptional activity.[36] However, this study also demonstrated a decreased body mass index in Ala12 allele carriers, a finding that has been not confirmed by a large meta-analysis.[4,36] Mouse models showed that mice with reduced *PPARγ2* activity seem to be resistant to high-fat diet-induced obesity, but not high-carbohydrate diet induced obesity.[37] The current study did not allow us to measure calorie intake of breastfed or bottle-fed children. Calorie intake in breastfeeding has been shown to be dependent of maternal factors, such as body composition and nutritional status.[38] All these factors indicate that the underlying mechanism in which this *PPARγ2* Pro12Ala interacts with early dietary intake is highly complex and emphasizes the necessity for further genetic association and functional studies.

In summary, our results suggest that this *PPARγ2* polymorphism influences growth rate from early fetal life to infancy. This effect on growth rate is restricted to infants who were breastfed for less than 4 months. Studies in larger cohorts with a longer follow-up period and will allow us to examine whether these effects persist throughout childhood. Additionally, systematic searches for common genetic variants by means of genome-wide association studies may enable us to obtain a more complete understanding of what genes are involved in growth in fetal life and infancy and how they interact with the environment.

Appendix

For weight, the best-fitting models can be written as:
Overall: Weight (grams) $= \beta_0 + \beta_1 * age + \beta_2 * age^2 + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 * gender.$
Fetal: Weight (grams) $= \beta_0 + \beta_1 * age^{-2} + \beta_2 * age^{-2} * ln(age) + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 * age^{-2} * (ln(age))^2 + \beta_6 * gender.$
Infancy: Weight (grams) $= \beta_0 + \beta_1 * age + \beta_2 * age^{-0.5} + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 *$ *gestational age at birth* $+ \beta_6 *$ *gender.*

Since body length cannot be measured in fetal life, repeated measures regression analysis was performed over the period from 1.5 to 18 months. The best-fitting model is:
Infancy: Length (cm) $= \beta_0 + \beta_1 * age + \beta_2 * age^{-2} + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 *$ *gestational age at birth* $+ \beta_6 *$ *gender.*

Since head circumference was not measured at birth, we used the head circumference measured at 1.5 months as a proxy:
Overall: Head circumference (cm) $= \beta_0 + \beta_1 * age + \beta_2 * ln(age) + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 * gender.$
Fetal: Head circumference (cm) $= \beta_0 + \beta_1 * age^2 + \beta_2 *$ $age^2 * ln(age) + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 * gender.$
Infancy: Head circumference (cm) $= \beta_0 + \beta_1 * age + \beta_2 * age^{0.5} + \beta_3 * genotype + \beta_4 * genotype * age + \beta_5 *$ *gestational age at birth* $+ \beta_6 *$ *gender.*

In these models, the term including 'β_0' reflects the intercept and the term including 'β_1 and β_2 (and β_5 in the model for fetal weight)' reflects the slope of growth in grams or centimeter per week for the reference group (Pro12Pro individuals). All models were adjusted for gender ('β_5' or 'β_6') and models for growth during infancy were additionally adjusted for gestational age at birth ('β_5'). The terms including 'β_3' and 'β_4' reflect the age independent differences and the growth differences between the genotypes, respectively.

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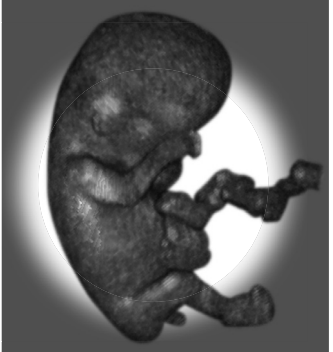
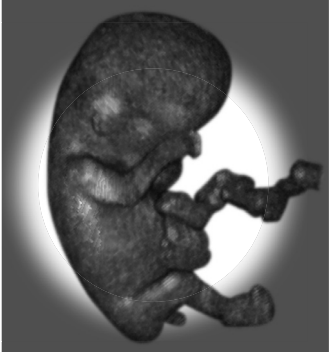


CHAPTER 4

Genome-wide association studies on early growth



CHAPTER 4.1

Variants at two loci (in *ADCY5* and near *CCNL1*) influence fetal growth and birth weight

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Abstract

Aims and Methods: To identify genetic variants associated with birth weight, we meta-analyzed six genome-wide association (GWA) studies (N=10,623 Europeans from pregnancy/birth cohorts) and followed up two lead signals in thirteen replication studies (N=27,591).

Results and Conclusions: Rs900400 near *LEKR1* and *CCNL1* (P=2x10^{-35}), and rs9883204 in *ADCY5* (P=7x10^{-15}) were robustly associated with birth weight. Correlated SNPs in *ADCY5* were recently implicated in regulation of glucose levels and type 2 diabetes susceptibility,[1] providing evidence that the well described association between lower birth weight and subsequent type 2 diabetes[2,3] has a genetic component, distinct from the proposed role of programming by maternal nutrition. Using data from both SNPs, the 9% of Europeans with 4 birth weight-lowering alleles were, on average, 113g (95%CI 89-137g) lighter at birth than the 24% with 0 or 1 allele (P_{trend}=7x10^{-30}). The impact on birth weight is similar to that of a mother smoking 4-5 cigarettes per day in the third trimester of pregnancy.[4]

Introduction

The extremes of birth weight are associated with high risks of perinatal morbidity and mortality.[5,6] In addition, there are well-documented observational associations between lower birth weight and later life chronic disease, including type 2 diabetes, cardiovascular disease and higher blood pressure.[2,3] The mechanisms underlying these associations are poorly understood. Birth weight is a complex multifactorial trait.[7,8] The importance of genetic factors acting independently of the intra-uterine environment is illustrated by correlations between paternal height or weight and offspring birth weight,[7,9,10] and genetic variants that are associated both with low birth weight and increased risk of type 2 diabetes may account for some of the observed correlation between these phenotypes.[11-13] However, the genetic loci that influence birth weight are largely unknown.

Birth weight may be influenced directly by fetal genotype, and also indirectly by maternal genotype operating through the intra-uterine environment. This is clearly illustrated by observations of mothers and offspring with rare, heterozygous glucokinase (*GCK*) mutations. By reducing insulin secretion, these mutations increase offspring birth weight by 600g when inherited by the mother and reduce birth weight by 530g when inherited by the fetus.[14]

To search for common genetic variants associated with birth weight, we performed a meta-analysis of GWA studies. We reasoned that finding such variants, even those with modest effects, would lead to enhanced understanding of pathways important for fetal growth and those underlying the associations between fetal growth and adult disease.

Methods

Stage 1: GWA meta-analysis of birth weight

Discovery samples, genotyping and imputation

We selected six population-based European studies with birth weight, gestational age and GWA data available by the beginning of May 2009 (combined N=10,623): the Northern Finland 1966 Birth Cohort (NFBC1966; N=4333); Netherlands Twin Register (NTR; N=414; singletons only); and sub-samples from the 1958 British Birth Cohort (B58C-WTCCC, N=1227; B58C-T1DGC, N=2037), Generation R (N=1194) and Avon Longitudinal Study of Parents And Children (ALSPAC; N=1418). The B58C-WTCCC and B58C-T1DGC were analyzed separately because they were genotyped on different platforms at different times. However, there is no systematic phenotypic difference between these subsamples. Genotypes were obtained using high-density SNP arrays, and then imputed for ~2.4 million HapMap SNPs (Phase II, release 21/22, http://hapmap.ncbi.nlm.nih.gov/).

Statistical analysis within discovery samples

Multiple and preterm births (gestational age <37 weeks) were excluded from all analyses. Birth weight was transformed into a Z-score (= [value-mean]/SD) to allow comparison of the data across studies. The overall (as opposed to sex-stratified) mean and SD from each study were used to create Z-scores. The association between each SNP and birth weight was assessed in each study sample using linear regression of birth weight Z-score against genotype (additive model), with sex and gestational age as covariates. Imputed genotypes were used only where directly-assayed genotypes were unavailable. In addition to this "UNIFORM" analysis, a second analysis ("BEST") was performed, in which the analysis details were decided within each study.

Meta-analysis of discovery samples

Data exchange was facilitated by the SIMBioMS platform (simbioms.org).[15] Prior to meta-analysis, SNPs with a minor allele frequency <1% and poorly-imputed SNPs (proper_info ≤0.4 [SNPTEST]; r^2 ≤0.3 [MACH2QTL]) were filtered. Fixed effects meta-analyses of the UNIFORM and BEST analyses were each run in parallel in two different study centers. Each was performed using different software packages: METAL (http://www.sph.umich.edu/csg/ abecasis/metal/index.html); and MetaMapper (developed in-house at Imperial College London, UK). Genomic control[16] was applied twice at the meta-analysis stage: first, to adjust the statistics generated within each cohort; and second, to adjust the overall meta-analysis statistics (λ=1.032). The results from the UNIFORM analysis were meta-analyzed using the inverse-variance method, whereas for the BEST analysis a Z-score weighted method that allows for differences in the units of beta coefficients and standard errors was applied.[17] SNPs available for less than half of the total expected sample were excluded. Final meta-analysis results were obtained for 2,427,548 SNPs. Those SNPs that reached a p-value threshold of $<10^{-7}$ in the UNIFORM analysis (N=10 SNPs, representing 2 distinct genomic regions on chromosome 3 were considered for further follow-up. The UNIFORM (reported here) and BEST analyses (data not shown) gave very similar results.

Checking for independent associations at the two loci

To test for the presence of additional association signals around the most strongly associated SNP in each region (rs900400 and rs9883204), we re-ran the UNIFORM association analysis on chromosome 3 in each discovery sample, including rs900400 and rs9883204 genotypes as additional covariates. Where these SNPs were imputed, genotype dosage was calculated from the genotype probabilities and used in the model. We meta-analyzed results using the inverse-variance method.

Stage 2: Follow-up of two lead signals in additional samples

Follow-up samples, genotyping and analysis

We used 17 study samples (combined N=30,098) to follow up the two lead signals from the GWA meta-analysis (represented by index SNPs rs900400 and rs9883204). Thirteen of the samples (combined N=27,591) were of European ancestry and were used for replication of the birth weight associations. We also examined associations in four further non-European or admixed study samples (combined N=2507). Informed consent was obtained from all discovery and follow-up study participants (or parental consent, as appropriate), and study protocols were approved by the local ethics committees. If the index SNP was unavailable, a closely correlated proxy was substituted (rs1482853 or rs900399 for rs900400 [HapMap r^2=1 and 0.96, respectively]; rs2877716 or rs6798189 for rs9883204 [HapMap r^2=0.95 and 0.93, respectively]). In four of the replication studies, the index SNPs were imputed from genome-wide genotype data. The UNIFORM birth weight analysis (described above) was performed within each study sample. To investigate whether the associations were similar in the sexes, we repeated the analysis in males and females separately.

Meta-analyses

We performed fixed effects inverse variance meta-analyses of the UNIFORM results as follows: (i) including all 13 European replication samples; (ii) including all 19 discovery and replication samples of European descent, (iii) a sensitivity analysis, excluding the three studies without gestational age; and (iv) including all 23 study samples, regardless of ethnic background. We meta-analyzed the sex-stratified results from all European studies. All meta-analyses were performed in parallel at two different study centers, using different software packages (the METAN module, developed for Stata v.10,[18] Meta-Analyst [Beta 3.13],[19] RMeta in R [v.2.7.0]). We used the Cochran Q test and the I^2 statistic[20] to assess evidence of between-study heterogeneity of effect sizes.

Analysis of additional phenotypes

Birth length, birth head circumference, ponderal index and small for gestational age

Where available, we created Z-scores (value-mean/SD) within each study for birth length, head circumference and ponderal index (birth weight/length^3). We used linear regression to assess the association between each outcome and each SNP (rs900400 or rs9883204, or proxies), with sex and gestational age as covariates. To examine the odds of small-for-gestational age (SGA), we created sex- and gestational age-adjusted birth weight Z-scores (SDS) within 15 of the available European studies using Growth Analyser 3.0 (http://www.growthanalyser.org; Dutch Growth Research Foundation, Rotterdam,

the Netherlands). The reference was a cohort of 475,588 children born between 1977 and 1981 in Sweden.[21] Subsequently, each study defined SGA as below the 10th percentile of birth weight SDS within their study population. We analysed the associations between the two top hits and SGA using logistic regression. Analyses were repeated with a 5th percentile cut-off. We combined the results across studies using fixed effects inverse variance meta-analysis.

Combined allele score

To estimate the birth weight effect sizes attributable to the two loci in combination, we created an allele score using information from both SNPs, which was weighted by effect size. This allowed us to estimate the differences in birth weight between individuals with different numbers of birth weight-lowering alleles at the two loci. We used nine European replication samples in which gestational age was available (N=20,190). After verifying that the two SNPs were statistically independent, we generated the score using the formula

$$s_j = 2 \times \sum_{i=1}^{2} w_i g_{ij} \Big/ \sum_{i=1}^{2} w_i$$

where s_j is score for individual j, g_{ij} is number of birth weight-lowering alleles (0, 1, 2) for SNP i carried by individual j and w_i is effect size for SNP i from the UNIFORM analysis within the cohort. We performed linear regression of birth weight (grams) against the allele score (additive model), with sex and gestation as covariates. We combined the coefficients from the nine studies using fixed effects inverse variance meta-analysis. We then rounded the weighted score to the nearest integer, grouping scores "0" and "1" together, and performed linear regression of birth weight against the rounded score as indicator variables, with sex and gestation as covariates. The beta coefficients from the comparison of score 4 versus 0/1 in all nine studies were meta-analyzed (inverse variance, fixed effects).

Variance explained

To estimate the percentage of variation in birth weight explained by each of the associated loci, we obtained the adjusted-R^2 from univariate linear regression of birth weight against genotype. We then calculated a mean value from all European studies, weighted by sample size. For comparison, we also calculated the variance explained by variables such as gestational age, maternal age and smoking.

Analyses of potential confounders

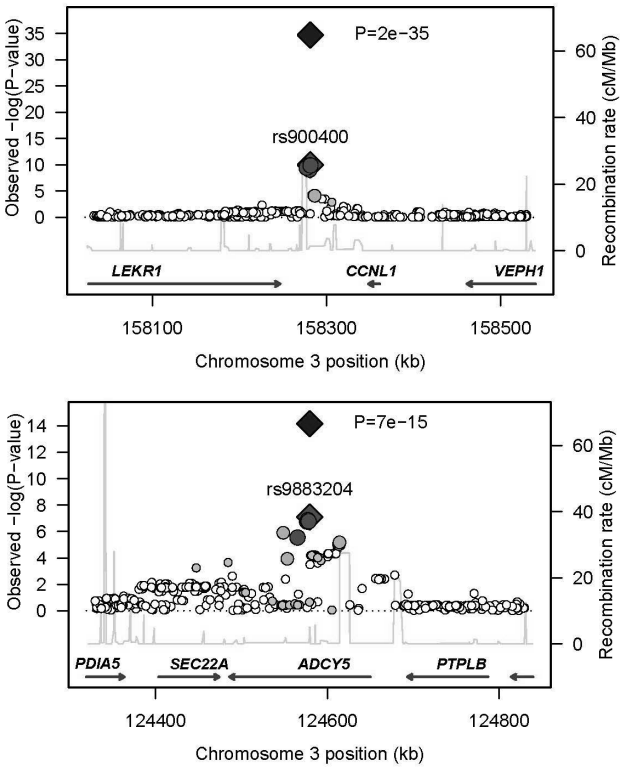
To assess whether the associations were independent of maternal genotype, we used mother-offspring pairs from the 5 studies with both maternal and fetal genotype available (total N=8880 for rs900400; N=9127 for rs9883204). Within each study, we performed the UNIFORM analysis, with maternal genotype as an additional covariate. For direct comparison, we repeated this without maternal genotype, using only subjects for whom maternal genotype was available. We performed two inverse variance meta-analyses (fixed effects) for each SNP, combining regression coefficients for (i) fetal genotype, and (ii) fetal genotype adjusted for maternal genotype.

To verify that the SNPs were not associated with maternal covariates of birth weight which could theoretically confound the observed associations with birth weight (including maternal age, BMI, parity, smoking, pre-eclampsia, education), we used linear or logistic regression to model the association between each covariate and genotype, using nine European replication cohorts with gestational age available. Where possible, we meta-analyzed results to assess overall evidence of association.

Results and discussion

We meta-analyzed association statistics from 2,427,548 directly-genotyped and imputed SNPs in singletons of European descent from six discovery GWA studies (N=10,623). Birth weight (BW) was standardized to Z-scores within each study ([BW-mean]/standard deviation, SD) and adjusted for sex and gestational age. We observed SNPs at two independent loci on chromosome 3 that were associated with birth weight at, or close to, genome-wide significance ($P<5x10^{-8}$). The first locus was at 3q25, between *CCNL1* and *LEKR1*; and the second, at 3q21 in *ADCY5* (Figure 1). To replicate these associations, we genotyped the most strongly associated SNP from each locus (rs900400 from 3q25; rs9883204 from 3q21), or a closely-correlated proxy (HapMap r^2=0.927-0.963), in thirteen further samples of European descent (N=27,591). Robust evidence of association was seen for both signals in these replication samples (Figure 2; $P=3x10^{-26}$ and $3x10^{-9}$, respectively). Combining all discovery and replication samples, each additional C-allele of SNP rs900400 (frequency 32-47%) was associated with a 0.086 SD lower birth weight (95%CI: 0.073-0.100; $P=2x10^{-35}$), while each C-allele of SNP rs9883204 (frequency 71-83%) was associated with a 0.063 SD lower birth weight (95%CI: 0.047-0.079; $P=7x10^{-15}$; Table 1). These SD changes equate approximately to differences of 40g and 30g per allele, respectively (median study SD=484g). Analysis conditional on the index SNPs, rs900400 and rs9883204 did not produce any evidence for additional independent signals at either of the loci.

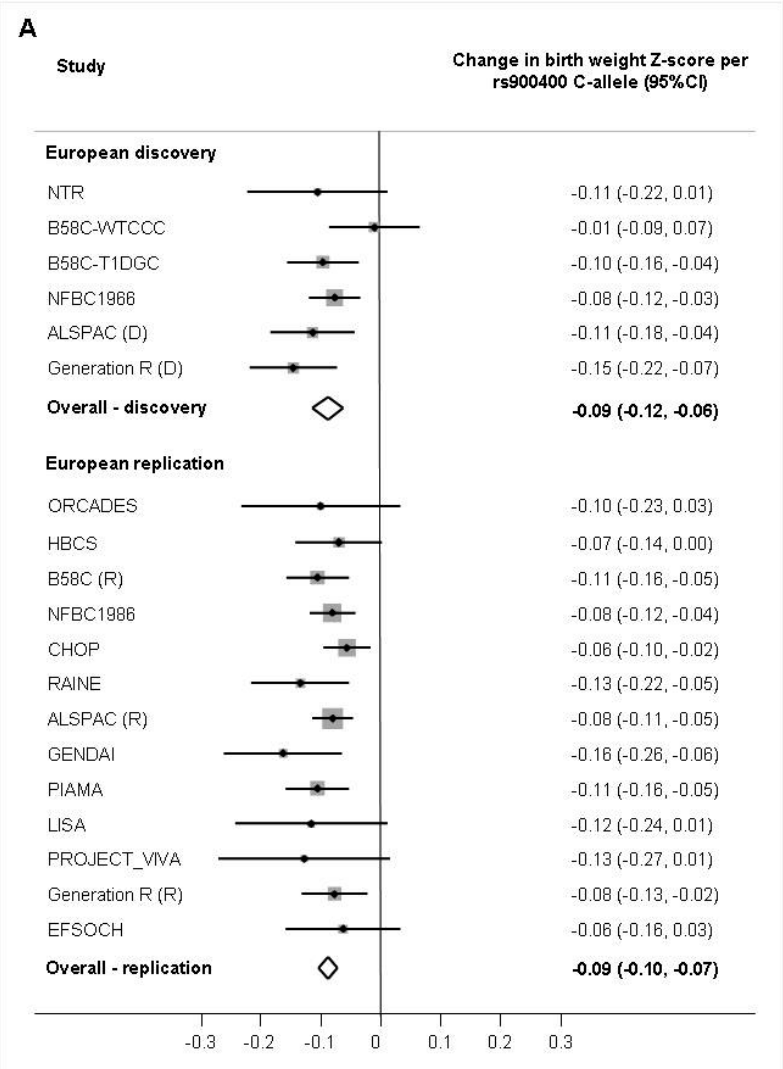
Figure 1: Regional plots of two novel associations with birth weight.



For each of the two regions, 3q25 ***[A]*** *and 3q21* ***[B]****, directly genotyped and imputed SNPs are plotted using filled circles with their meta-analysis P values (as -log$_{10}$ values) as a function of genomic position (NCBI Build 35). In each plot, the discovery stage SNP taken forward to replication stage is represented by a blue diamond (defining a global meta-analysis P value), with its discovery meta-analysis P value denoted by a red diamond. Local LD structure is reflected by the plotted estimated recombination rates (taken from HapMap) in the region around the associated SNPs and their correlated proxies. Each analyzed SNP is represented by circle. The colour scheme of the circles respects LD patterns (HapMap CEU pair-wise r2 correlation coefficients) between top discovery SNP and surrounding variants: white r2<0.2, grey 0.5> r2 >= 0.2, orange 0.8> r2 >= 0.5, red r2 >= 0.8. Gene annotations were taken from the University of California Santa Cruz genome browser.*

We found no evidence of heterogeneity between the studies examined (P>0.5; I^2=0%),[20] despite differences in mean birth weight (reflecting secular and population differences in birth weight distribution; Table 2), and the associations with birth weight were similar in males and females (P>0.05 for difference in effect sizes). Gestational age was not available as a covariate in three of our replication studies (combined N=6235), but these studies did not introduce detectable heterogeneity, and their removal from the meta-analysis changed the results very little (Figure 2 and Table 1 footnote). We also assessed the effects of the two SNPs on birth weight in a limited number of non-European or admixed samples from 2 studies (N=1415 Filipino subjects from the Cebu

Figure 2a: Forest plots of the association between birth weight and genotype at each locus.

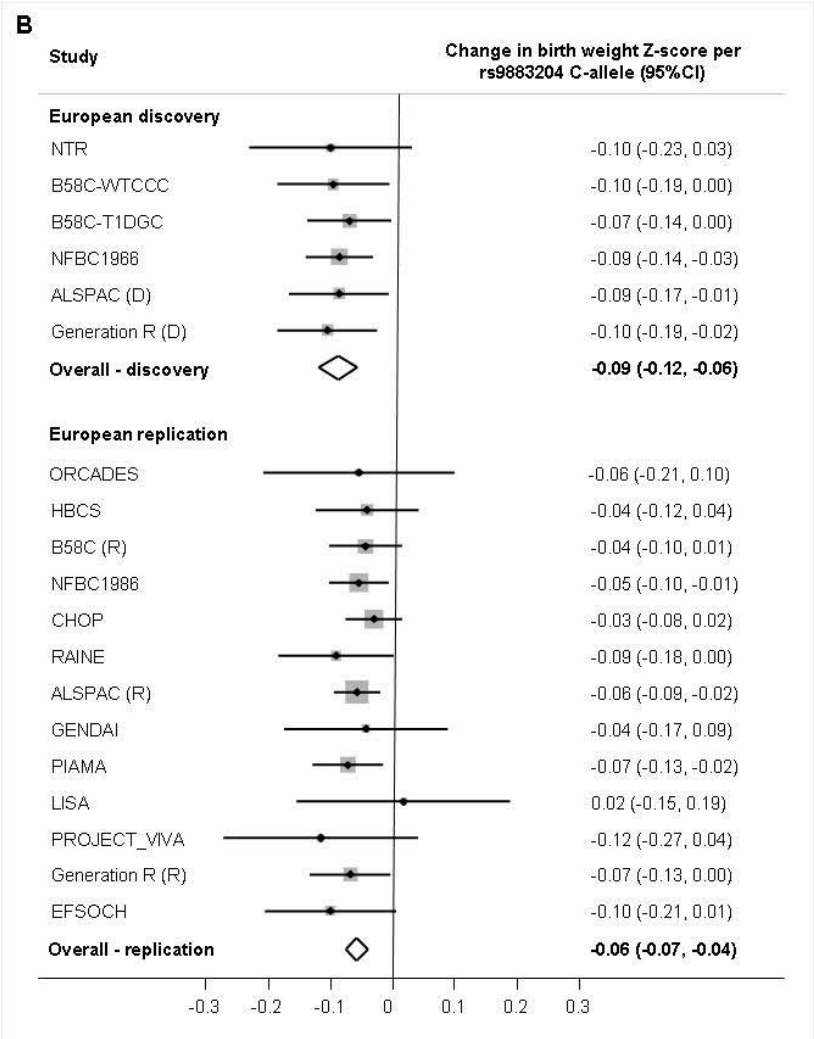


[A] *Index SNP rs900400 at 3q25.*
If the index SNP was unavailable, a closely-correlated proxy (HapMap r^2>0.9) was used.

Longitudinal Health and Nutrition Survey, and N=298-448 Black, Moroccan and Turkish subjects from Generation R; data not shown). There was no difference in the effect sizes observed relative to the European studies (*P*>0.5), but power to detect association was limited. Further well-powered studies will be needed to investigate these associations in non-Europeans.

Maternal and fetal genotypes are correlated due to segregation. In a previous study, an observed association between fetal *TCF7L2* genotype and birth weight was driven by

Figure 2b: Forest plots of the association between birth weight and genotype at each locus.



[B] *Index SNP rs9883204 at 3q21.*
If the index SNP was unavailable, a closely-correlated proxy (HapMap r^2>0.9) was used.

the effects of maternal *TCF7L2* variation on the intra-uterine environment, rather than a direct effect on fetal growth.[22] To distinguish between these two mechanisms, we tested whether the novel birth weight associations were independent of maternal genotype. We genotyped both SNPs in all available maternal DNAs (N=9127; 5 study samples). Meta-analysis of associations between birth weight and fetal genotype, conditional on maternal genotype, yielded results which were very similar to the original associations at both loci (data not shown), showing that these are direct fetal effects. As expected,

Table 1: Associations between novel birth weight loci and anthropometric traits at birth

Phenotype	Locus 3q25, nearest genes: *CCNL1*, *LEKR1*[a]				Locus 3q21, *ADCY5*[b]			
	N	Effect	95%CI	*P* value[c]	N	Effect	95%CI	*P* value[c]
Birth weight Z-score[d]	37745	-0.086 SD	-0.100, -0.073	$2x10^{-35}$	38214	-0.063 SD	-0.079, -0.047	$7x10^{-15}$
Birth length Z-score	21512	-0.028 SD	-0.046, -0.010	0.002	21782	-0.044 SD	-0.066, -0.022	$4x10^{-5}$
Birth head circumference Z-score	17349	-0.024 SD	-0.044, -0.004	0.017	17693	-0.025 SD	-0.048, -0.004	0.030
Ponderal index[e] Z-score	21515	-0.094 SD	-0.113, -0.074	$5x10^{-21}$	21785	-0.032 SD	-0.055, -0.009	0.006
Odds ratio for SGA <10th percentile[f]	30370	1.16	1.10, 1.23	$1x10^{-7}$	30778	1.09	1.02, 1.16	0.009

Results are from inverse variance, fixed effects meta-analysis of all 19 study samples of European ancestry. The effect allele for each SNP is labelled on the positive strand according to HapMap. The effect is the beta coefficient (or odds ratio) for genotype, assuming an additive genetic model. If the index SNP was unavailable, this was substituted with a closely-correlated (HapMap r^2>0.9) proxy (rs1482853 or rs900399 for rs900400; rs2877716 or rs6798189 for rs9883204). There was no evidence of between-study heterogeneity of effect size estimates (all P>0.18; I^2<26%). [a]Index SNP rs900400, effect allele C (40% frequency in HapMap CEU; range 32-47% in our European study samples). [b]Index SNP rs9883204, effect allele C (73% frequency in HapMap CEU; range 71-83% in our European study samples). [c]The P value for the birth weight meta-analysis includes the double-GC correction of the discovery meta-analysis. [d]Excluding the three studies that were unable to adjust for gestational age, the beta (s.e.m.), N and P values in the birth weight analysis were: -0.089(0.008), N=31510, P=$7x10^{-32}$ (3q25); -0.068(0.009), N=31901, P=$8x10^{-15}$ (3q21). [e]Ponderal index = birth weight/length^3. [f]SGA <5th percentile, OR (P value): rs900400=1.11 (0.004); rs9883204=1.04 (0.41).

there was no association between fetal genotype and various covariates of birth weight that were not included in our main analysis (maternal smoking, BMI, parity, education, age at delivery; all *P*>0.05; data not shown).

Birth weight may be influenced by skeletal growth or fat mass. In available samples, we analyzed the association between each locus and birth length, birth head circumference and ponderal index (Table 1). The association with ponderal index, relative to the birth length and head circumference associations, was particularly strong for the rs900400 SNP (0.094 SD [95%CI: 0.074-0.113] per C-allele; *P*=$5x10^{-21}$), suggesting a greater association with fat mass than skeletal growth. For the rs9883204 SNP, the measures showed more proportionate changes (Table 1). We investigated associations with adult height and BMI using published GWA meta-analyses from the GIANT consortium.[23,24] Only the rs900400 signal was captured in the published height data at r^2>0.8 (since that study only included direct genotypes from the Affymetrix Genechip 500k), and there was no association (*P*=0.64; N=9818). There was no association with adult BMI for either locus (N≈32500, P>0.1). This is consistent with the weak observational association between

Table 2: Mean birth weight (SD) by genotype and individual association results by study

Study type	Study	Year(s) of birth	Total N[a]	% male	Locus 3q25, nearest genes: *CCNL1*, *LEKR1*[b]				Locus 3q21, *ADCY5*[b]			
					TT	CT	CC		TT	CT	CC	
					Mean BW in g (SD)	Mean BW in g (SD)	Mean BW in g (SD)	*P* value[c]	Mean BW in g (SD)	Mean BW in g (SD)	Mean BW in g (SD)	*P* value[c]
Discovery	NTR	1923-86	414	37.9	3470 (652)	3401 (615)	3329 (646)	0.08	3500 (720)	3402 (604)	3359 (633)	0.09
	B58C-WTCCC	1958	1227	50.4	3367 (444)	3337 (455)	3364 (454)	0.77	3459 (457)	3357 (456)	3336 (455)	0.05
	B58C-T1DGC	1958	2037	49.2	3399 (468)	3339 (464)	3308 (461)	$1x10^{-3}$	3396 (463)	3375 (484)	3341 (463)	0.07
	NFBC1966	1966	4333	48.1	3567 (458)	3519 (458)	3503 (458)	$5x10^{-4}$	3630 (459)	3559 (459)	3529 (459)	$4x10^{-3}$
	ALSPAC (D)	1991-2	1418	48.8	3486 (481)	3419 (482)	3374 (467)	$2x10^{-3}$	3451 (458)	3462 (465)	3405 (514)	0.03
	Generation R (D)	2002-6	1194	53.1	3633 (435)	3562 (447)	3492 (448)	$1x10^{-4}$	3655 (449)	3593 (444)	3549 (456)	0.01
Replication	ORCADES	1920-88	328	43.3	3635 (599)	3615 (594)	3487 (602)	0.12	3542 (612)	3670 (605)	3566 (595)	0.74
	HBCS	1934-44	1566	42.7	3462 (436)	3434 (438)	3403 (430)	0.06	3391 (426)	3479 (434)	3431 (418)	0.33
	B58C (R)	1958	2550	51.6	3407 (454)	3341 (451)	3308 (456)	$7x10^{-5}$	3338 (457)	3387 (448)	3340 (477)	0.14
	NFBC1986	1985-6	5008	49.1	3656 (440)	3607 (440)	3591 (440)	$4x10^{-5}$	3674 (441)	3646 (441)	3620 (441)	0.03
	CHOP	1987-2009	5149	53.3	3384 (634)	3333 (646)	3318 (628)	$5x10^{-3}$	3389 (641)	3357 (647)	3341 (609)	0.19
	RAINE	1989-92	988	52.4	3507 (428)	3432 (417)	3384 (429)	$1x10^{-3}$	3472 (426)	3489 (431)	3427 (425)	0.06
	ALSPAC (R)	1991-2	5695	54.6	3303 (547)	3259 (568)	3229 (493)	$3x10^{-6}$	3305 (464)	3288 (580)	3257 (626)	$3x10^{-3}$
	GENDAI	1994-6	758	45.5	3401 (530)	3215 (528)	3235 (529)	$1x10^{-3}$	3291 (539)	3286 (539)	3260 (539)	0.53
	PIAMA	1996-7	1789	51.3	3629 (438)	3575 (443)	3512 (427)	$9x10^{-5}$	3619 (441)	3607 (425)	3554 (430)	0.01
	LISA	1998-9	387	56.9	3476 (366)	3454 (363)	3368 (363)	0.07	3532 (365)	3429 (366)	3443 (367)	0.84
	PROJECT VIVA	1999-2003	300	50.0	3711 (406)	3646 (411)	3594 (407)	0.08	3698 (412)	3703 (402)	3625 (408)	0.15
	Generation R (R)	2002-6	1885	50.3	3558 (435)	3527 (423)	3481 (413)	$6x10^{-3}$	3615 (433)	3534 (435)	3518 (430)	0.04
	EFSOCH	2003-4	719	53.1	3556 (427)	3509 (432)	3504 (431)	0.20	3660 (433)	3513 (435)	3503 (432)	0.07

BW, birth weight. All birth weight values are adjusted for sex and gestational age. [a]*Study N in the birth weight association analysis for rs900400 genotype. Total numbers of European discovery and replication samples, respectively, were N=10623 and N=27122 for rs900400; N=10623 and N=27591 for rs9883204.* [b]*If the index SNP was uZnavailable, this was substituted with a closely-correlated (HapMap r^2>0.9) proxy (rs1482853 or rs900399 for rs900400 at 3q25; rs2877716 or rs6798189 for rs9883204 at* 3q21). [c]*P* value is from linear regression of birth weight Z score against SNP (additive model), with sex and gestational age as covariates. All study samples were of European descent.

Key to study names: NTR, Netherlands Twin Register; B58C-WTCCC, British 1958 Birth Cohort – Wellcome Trust Case Control Consortium subset; B58C-T1DGC, British 1958 Birth Cohort – Type 1 Diabetes Genetics Consortium subset; NFBC1966, Northern Finland Birth Cohort 1966; ALSPAC (D), Avon Longitudinal Study of Parents and Children Discovery subset; Generation R (D), Generation R Discovery subset; ORCADES, Orkney Complex Disease Study; HBCS, Helsinki Birth Cohort Study; B58C (R), British 1958 Birth Cohort Replication subset; NFBC1986, Northern Finland Birth Cohort 1986; CHOP, Children's Hospital Of Philadelphia; RAINE, The Raine Study; ALSPAC (R), Avon Longitudinal Study of Parents and Children Replication subset; GENDAI, GENe and Diet Attica Investigation; PIAMA, Prevention and Incidence of Asthma and Mite Allergy; LISA, Lifestyle – Immune System – Allergy; Project Viva, The Project Viva Cohort; Generation R (R), Generation R Replication subset; EFSOCH, Exeter Family Study Of Childhood Health.

birth weight and adult BMI,[25] indicating that they are largely governed by different processes.

Although birth weight is a continuous trait, standard clinical cut-offs are used to identify neonates who are small for gestational age and who may require further observation. We therefore assessed whether each SNP increased the odds of gestational age-adjusted birth weight <10th percentile. Both loci were associated with smallness for gestational age: odds ratios (OR) 1.16 [95%CI: 1.10-1.23] (P=1x10^{-7}) and 1.09 [1.02-1.16] (P=0.009) per C-allele of rs900400 and rs9883204, respectively (Table 1).

The birth weight signal marked by rs900400 maps approximately 35kb 3-prime to the leucine, glutamate and lysine rich 1 (*LEKR1*) locus and 67kb 3-prime to cyclin L1 (*CCNL1*). Neither gene has previously been implicated in fetal growth. The CCNL1 protein may be involved in pre-mRNA splicing and RNA processing, and associates with cyclin-dependent kinases.[26] A non-coding RNA of unknown function, 682bp from rs900400 (AK311218, Human March 2006 Assembly 18), overlaps with the signal. We found no evidence for association at a genome-wide level (P>5x10^{-8}) between our 3q25 birth weight signal and mRNA expression in lymphocytes, using the publicly available 'mRNA by SNP Browser 1.0',[27] and there was no association between rs900400 or rs900399 and type 2 diabetes or related adult glycemic traits in the recent GWA meta-analysis from the MAGIC consortium (P>0.1).[1] A range of approaches (including resequencing and functional studies) will be required to establish which gene (*CCNL1*, *LEKR1* or another gene) is mediating the effect on fetal growth.

The second birth weight locus at 3q21 (index SNP rs9883204) maps within the adenylyl cyclase 5 gene (*ADCY5*). ADCY5 belongs to the family of enzymes responsible for the synthesis of cyclic adenosine monophosphate (cAMP).[28-30] Allele A of rs11708067, in linkage disequilibrium (LD) with the birth weight-lowering C-allele of rs9883204 (r^2=0.75), is associated with a higher risk of type 2 diabetes (OR: 1.12 [95%CI: 1.04-1.15]; P=9.9x10^{-21}; 40,655 cases/87,022 controls), higher fasting glucose (0.027 mmol/l [95%CI: 0.021-0.033]; P=7.1x10^{-22}; N=118,475), and reduced Homeostasis Model Assessment of steady-state beta-cell function (HOMA-B; P=7.1x10^{-12}; N=94,212),[1] suggesting that it may impact on insulin secretion. Fetal insulin is a key fetal growth factor, and these metabolic associations suggest that one mechanism explaining the *ADCY5* association with birth weight might be a direct effect of the fetal risk allele on fetal growth via reduced insulin secretion, consistent with the fetal insulin hypothesis.[11]

However, our previous studies suggest that an association between fetal genotype and birth weight is not characteristic of all type 2 diabetes loci. For example, susceptibility variants at *CDKN2A/B, IGF2BP2* and *SLC30A8* and *TCF7L2* were not associated with birth weight in previous studies of N>15000, after adjusting for maternal genotype.[12,22] To test this more comprehensively, we examined the associations between birth weight and all published/in-press type 2 diabetes (N=20) and fasting glucose (N=19) loci in our

discovery GWA meta-analysis (N=10,623).[1,31,32] Only *ADCY5* and the previously observed birth weight association at *CDKAL1*[12,13] showed evidence of association at $P<0.01$. One explanation for the variable effects of different type 2 diabetes susceptibility loci on birth weight is that they may influence beta-cell function at different points of the life course, with *ADCY5* having a more marked effect *in utero* than the other loci. However, other mechanisms could be partially or wholly responsible for the *ADCY5* association with birth weight including the regulation of placental glucose transporter expression,[33] vitamin B_2 uptake in the placenta[34] and the architecture and permeability of the materno-fetal placental barrier.[35]

The associations at 3q25 and 3q21 explained 0.3% and 0.1% of the variance in birth weight, respectively. Given that estimates of the fetal genetic contribution to birth weight from twin and family studies are generally between 10 and 40%,[36,37] the proportion of heritability explained may be up to ten times greater. The variance explained by the first locus is comparable to that of maternal age (0.5%). We used a weighted risk allele score to estimate the differences in birth weight attributable to combinations of birth weight-lowering alleles at both loci. The 9% of Europeans with 4 birth weight-lowering alleles were, on average, 113g (95%CI 89-137g) lighter at birth than the 24% with 0 or 1 allele (P for trend $=7\times10^{-30}$). For comparison, this effect on birth weight is similar to the impact of a mother smoking 4-5 cigarettes per day,[4] and is approximately one-third of the impact of the severe malnutrition of the Dutch Famine of 1944-45, during which pregnant women consumed, on average, <1000 calories/day.[38]

To conclude, we have identified novel, robust associations between fetal genotype and birth weight at loci near *CCNL1* and at *ADCY5*. The causal mechanisms are not yet known, but the *ADCY5* locus has pleiotropic effects on glucose regulation and type 2 diabetes in adulthood. This is robust evidence that the widely described association between lower birth weight and subsequent type 2 diabetes has a genetic component, distinct from the proposed role of programming by maternal nutrition. Further understanding of these associations will illuminate the biological pathways important for fetal growth and its relationship with adult diseases.

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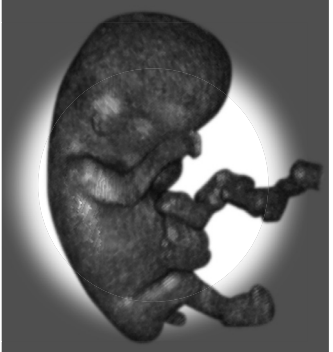

CHAPTER 4.2

Variants near *CCNL1/LEKR1* and in *ADCY5* and fetal growth patterns

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Abstract

Background: A recent genome-wide association study identified variants near *CCNL1/LEKR1* (rs900400) and in *ADCY5* (rs9883204) to be associated with birth weight. We examined the association of these variants with fetal growth characteristics in different trimesters, with a main interest in the timing of the associations and the affected body proportions.

Methods: We used data from two prospective cohort studies from fetal life onwards in the Netherlands and Australia. Repeated fetal ultrasound examinations were performed to measure head circumference (HC), abdominal circumference (AC), femur length (FL) and estimated fetal weight (EFW). Birth growth characteristics and placental weight were measured at birth. Analyses were based on a total group of 3,858 subjects with multiple observations.

Results: The C-allele of rs900400 was associated in second trimester with smaller fetal HC and FL (both $p<0.05$), and in third trimester with smaller HC, AC, FL and EFW (all p-values < 0.05). At birth, the C-allele of rs900400 was associated with all birth growth characteristics and placental weight. For each C-allele, the combined effect estimates were -1.66 (95% CI: -3.58, 0.26) grams and -19.2 (95% CI: -28.2, -10.3) grams for EFW in second and third trimester, respectively, and -60.8 (95% CI: -80.6, -41.1) grams for birth weight. The C-allele of rs9883204, was not associated with fetal growth in second trimester, but was associated with restriction of all growth characteristics, except HC, in third trimester and at birth. For each C-allele, the combined effect estimates were -1.13 (95% CI: -3.24, 0.98) grams and -18.5 (95% CI: -28.3, -8.6) grams for EFW in second and third trimester, respectively, and -48.1 (95% CI: -70.0, -26.1) grams for birth weight. We found a strong association with lower placental weight amongst C-allele carriers of rs9883204 (-16.40 (95% CI: -23.70, -9.17) grams).

Conclusions: Our results suggest that rs900400 is associated with slower fetal growth from early pregnancy onwards and a symmetric growth restriction, while rs9883204 leads to a slower fetal growth in third trimester and to asymmetric growth restriction. Both genetic loci were related with placental weight, but the genetic variant of *ADCY5* had the largest effect.

Introduction

Recently, common variants in two new independent loci on chromosome 3 have been identified to be associated with birth weight.[1] The first polymorphism (rs900400) is located between *LEKR1* and *CCNL1*, and the second (rs9883204) is in the *ADCY5* gene.[1] Of rs900400, no clear pathway in which this variant influences birth weight is evident. In contrast, variants in the *ADCY5* gene have been shown to influence glucose levels and insulin secretion.[2] Since insulin is the most important fetal growth hormone, genetically determined variation in insulin and glucose metabolism might be a plausible mechanism for influencing fetal growth.[3,4] These associations were a direct effect of the fetal genotype and not an indirect effect of the maternal genotype. The effect of having both variant C-alleles was a 80 grams lower birth weight.[1] However, birth weight alone might be an inappropriate measure of the individual growth potential since different fetal growth rates may lead to the same birth weight.[5] Also, birth weight is a summary estimate of both skeletal and non-skeletal growth. We have previously shown that rs900400 influences birth weight through a lower weight to length ratio, while rs9883204 influences birth weight through a smaller birth length. [1] Examining the associations of rs900400 and rs9883204 with fetal growth patterns and characteristics, such as head circumference, abdominal circumference, femur length and estimated fetal weight, may give more insight in how and during which period of pregnancy these common variants affect fetal growth and development. Symmetrical growth restriction, where all growth parameters are affected, is generally seen as decreased growth caused in early pregnancy.[6] In contrary, asymmetrical growth restriction, where the head size is relatively spared compared to decreased weight and length, is a phenomenon seen in late pregnancy.[6]

Therefore, we examined the associations of these two common variants on chromosome 3, rs900400 and rs9883204, with fetal growth patterns in different trimester of pregnancy among 3,858 subjects participating in two independent prospective cohort studies from early fetal life onwards.

Research design and methods

Design

This study was embedded in two prospective cohort studies from early fetal life onwards. These studies have been described previously in detail.[7-9] In brief, the Generaration R, is a population-based cohort study designed to identify early environmental and genetic causes of normal and abnormal growth, development and health in Rotterdam, the Netherlands. The cohort comprises 9,778 mothers and their children of different ethnici-

ties born in Rotterdam, the Netherlands and has been described in detail previously.[7,8] All children were born between April 2002 and January 2006. Fetal growth and their main determinants were repeatedly measured by fetal ultrasound, physical examinations, and questionnaires in each trimester of pregnancy. DNA was collected from cord blood samples. The Raine Study was started as a randomized controlled trial to evaluate the effects of repeated ultrasound in pregnant women in Perth, Western Australia. In total, 2,900 pregnant women were recruited between 1989 and 1991 prior to 18-weeks gestation at the King Edward Memorial Hospital (Perth, Western Australia). Women were randomized to repeated ultrasound measurements at 18, 24, 28, 34 and 38 weeks gestation or to a single ultrasound assessment at 18-weeks. DNA was collected at the year 14 follow-up. Both studies have been approved by the local Medical Ethics Committees. Written informed consent was obtained from all parents.

Population for analysis

Analyses were restricted to children from whom DNA was available for genotyping and with Caucasian ethnicity. In the Generation R Study, Caucasian ethnicity was defined as having principal components within four standard deviations of the CEU cluster of HapMap.[10] In the Raine Study, ethnicity was based on having at least one parent who identified him or herself by questionnaire as Caucasian (European decent). Anyone with at least one Aboriginal, Polynesian or Vietnamese parent was excluded from analysis. Analyses were based on a total group of 3,858 subjects (n = 2,661 in Generation R, n = 1,197 in Raine).

Genotyping

In the Generation R Study, cord blood for DNA isolation was available in 59% of all live-born participating children. Missing cord blood samples were mainly due to logistical constraints at the delivery. Individual genotype data were extracted from the genome-wide Illumina 610 Quad Array. Rs900399 was used as proxy for rs900400 (R^2: 1.000, D':1.000), and rs2877716 as a proxy for rs9883204 (R^2: 0.955, D':1.000). The genotype frequencies for rs900399 and rs2877716 were 17.4% / 47.0% / 35.7% (Hardy-Weinberg p=0.14) and 52.8% / 39.0% / 8.2% (Hardy-Weinberg p=0.17), respectively. In the Raine Study DNA was collected using standardized procedures from 74% of all adolescents who attended the 14 year follow-up on and a further 5% at the 16 year follow-up measurements. Genotype data was extracted from the genome-wide Illumina 660 Quad Array for each individual. The genotype frequencies for rs900399 and rs2877716 were 15.2% / 49.1% / 35.8% (Hardy-Weinberg p=0.43) and 56.6% / 37.7% / 5.8% (Hardy-Weinberg p=0.64), respectively.

Fetal growth characteristics

In both cohorts, fetal growth characteristics were measured to the nearest mm using standardized ultrasound procedures, which have been described in detail previously.[9,11,12] In the Generation R Study, fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) were measured to the nearest millimeter using standardized ultrasound procedures in first (median: 12.8 (90% range: 11.0-16.6) weeks), second (median: 20.3 (90% range: 19.1-22.0) weeks) and third (median: 30.1 (90% range: 29.0-32.0) weeks) trimester.[12] Estimated fetal weight (EFW) was calculated using the formula by Hadlock *et al.*[13] Birth weight, length and head circumference, placental weight, date of birth and sex were obtained from community midwife and hospital registries.

In the Raine Study, participating pregnant women attending the antenatal clinical at King Edward Memorial Hospital in Perth, Australia, were randomized into two groups. The first group (50%) had one routine ultrasound at mid-pregnancy (18 weeks), whereas the second group (50%) had additional repeated ultrasounds measurements at 24, 28, 34 and 38 weeks.[9] The measured fetal growth characteristics were the same as in the Generation R Study.[9]

Covariates

In both studies, information on maternal age, parity and weight before pregnancy was obtained by the first questionnaire at the enrolment in the study. Maternal smoking during pregnancy was assessed by multiple questionnaires during pregnancy. Maternal height was measured without shoes, and body mass index (weight/height^2 (kg/m^2)) was calculated. The prevalence of gestational diabetes was low in both studies (0.5% in Generation R and 4% in Raine). Since including gestational diabetes into the model did not materially change the effect estimates, this covariate was excluded from all analyses.

Data analysis

First, we used linear mixed effects models to assess the associations of rs900400 and rs9883204 with the longitudinal fetal growth patterns.[14] These models take into account the correlation between repeated measurements of the same subject, allow for incomplete outcome data, and enable assessing fetal growth patterns.[15] These models included gestational age and (gestational age)^2 as both fixed and random effects.[16] Sex was included as binary covariate. Consistent with previous statistical research in the Raine cohort,[17] ultrasound measurements were power transformed ($HC^{0.5}$, $AC^{0.4}$, $FL^{0.7}$, $\log_{10}$EFW) prior to analysis to ensure homoscedastic residuals. P-values and 95%

confidence intervals were calculated using Monte Carlo Markov Chain (MCMC) methods (100,000 simulations), which is conservative compared to the likelihood ratio test.

Secondly, we explored cross-sectionally the associations of the C-alleles of rs900400 and rs9883204 (used proxies: rs900399 and rs2877716, respectively) with growth parameters (head circumference, abdominal circumference, femur length, and estimated fetal weight) assuming an additive model using linear regression. For rs900400 the C-allele is the minor allele, whereas for rs9883204 the C-allele is the major allele. Cross-sectional analyses were performed at three time-points during pregnancy: first trimester (12-16 weeks of gestation), second trimester (16-24 weeks of gestation and third trimester (26-32 weeks of gestation). Only one measurement per subject was included in each time window. Similar models were used to assess the associations of rs900400 and rs9883204 with growth characteristics at birth (head circumference, length, weight, ponderal index (weight/length^3) and placental weight). All models were adjusted only for sex and gestational age since population genotype distribution is assumed to be unrelated to covariates, and the effect estimates were not materially affected by adjusting for covariates such as maternal age, pre-pregnancy body mass index or parity.[18] Combined effect estimates were calculated using fixed effects meta-analyses.

No adjustments were made for multiple testing due to the highly correlated nature of the outcome measurements. All effect estimates are presented with their 95% confidence interval (95% CI). All analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA) and the statistical graphics software R version 2.10.0.[19]

Results

The minor allele frequencies for rs900400 and rs9883204 in both cohorts were similar to those quoted in HapMap (rs900400: C=0.40, rs9883204: T=0.25).[10] Table 1 demonstrates no differences between the cohorts of the distribution of maternal and birth characteristics.

Figures 1-4 give the associations of for rs900400 and rs9883204 with longitudinally measured fetal growth patterns in the Generation R Study and Raine Study. Figures 1 and 2 show that the C-allele of rs900400 was consistently associated with decreased fetal growth of HC (both cohorts, $p<0.05$), AC (both cohorts, $p<0.005$), FL (both cohorts, $p<0.05$) and EFW (both cohorts, $p<0.05$), as compared to the T-allele. At 38 weeks of gestation in the Raine Study the CC genotype was associated with a head circumference, abdominal circumference and a femur length that was approximately 6 percentiles smaller than the TT genotype on clinical percentile charts. These estimates were slightly smaller at 32 weeks gestation but were consistent in both studies. In the longitudinal analyses for rs9883204, the C-allele was associated with all decreased fetal growth of all

Table 1: Subject characteristics.

Maternal Characteristics	Generation R (n=2,661)	Raine (n=1,197)
Age (years)	31.6 (4.2)	28.5 (5.8)
Height (cm)	170.9 (6.4)	164.1 (6.6)
Weight before pregnancy (kg)	68.5 (55.0 – 94.4)	58.0 (46.0 – 85.0)
Body mass index before pregnancy (kg/m2)	23.3 (19.5 – 32.0)	21.5 (17.8 – 31.6)
Parity (% nulliparous)	59.2%	86.4%
Birth characteristics		
Sex (% boys)	51.0%	52.8%
Gestational age (weeks)	40.3 (37.4 – 42.1)	39.1 (36.4 – 41.9)
Head circumference (cm)	34.1 (1.6)	34.6 (1.6)
Length (cm)	50.6 (2.3)	49.7 (2.4)
Weight (grams)	3544 (523)	3361 (560)
Ponderal index (kg/m^3)	27.52 (3.22)	27.3 (2.7)
Placental weight (grams)	646 (144)	594 (124)

Values represent mean (SD), medians (90% range) or percentages.

Figure 1a-d: Longitudinal association of rs900400 genotype with fetal growth in Generation R.

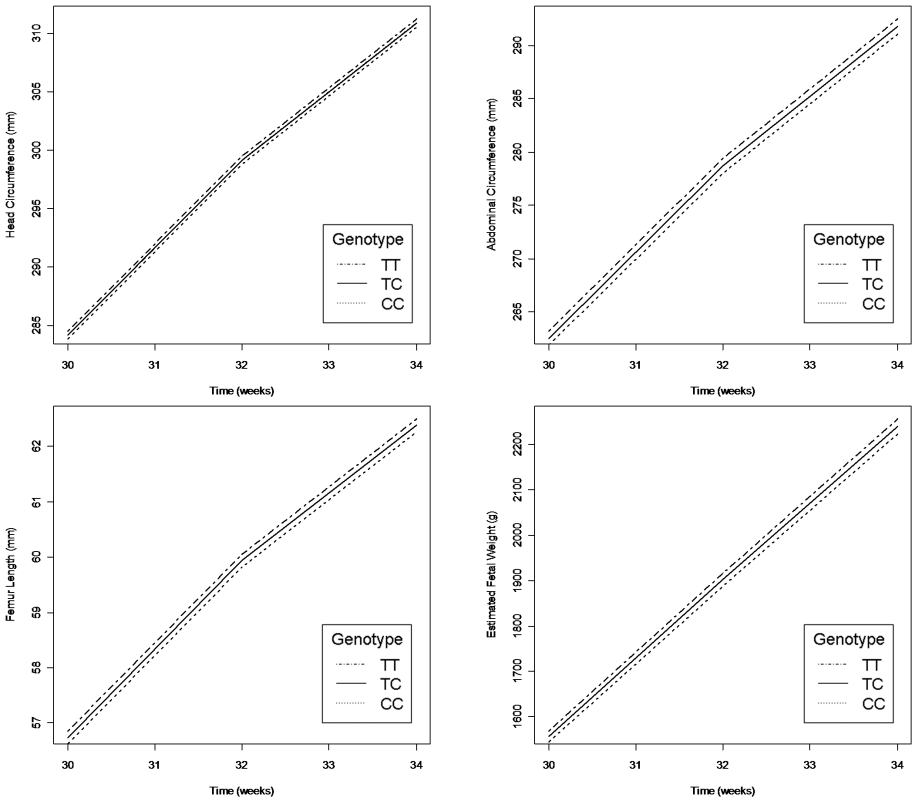
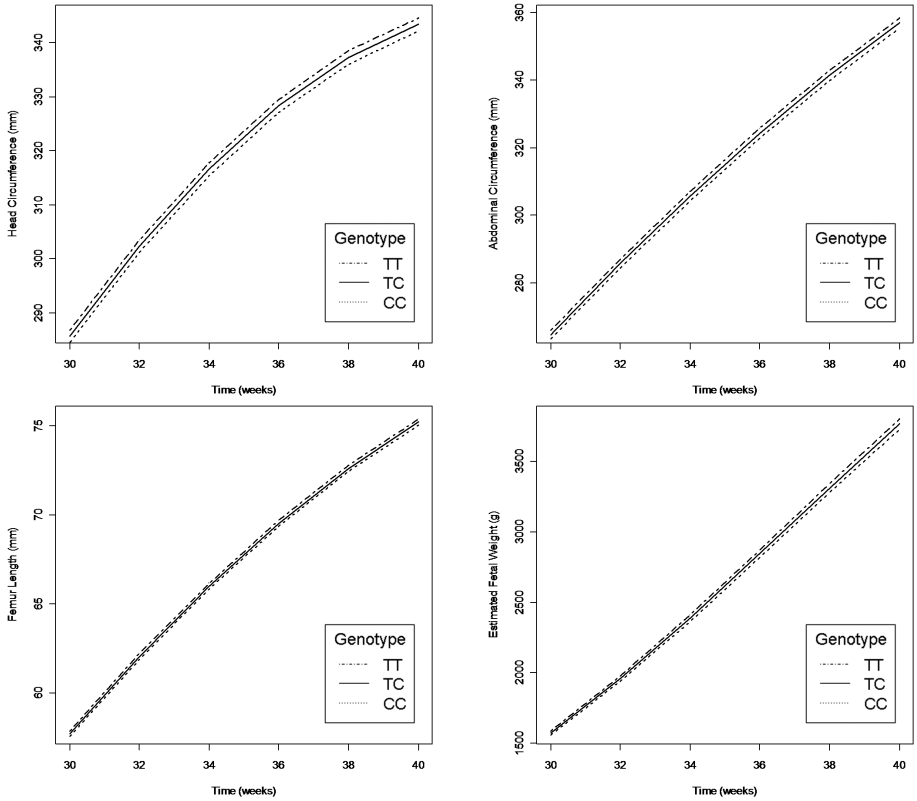


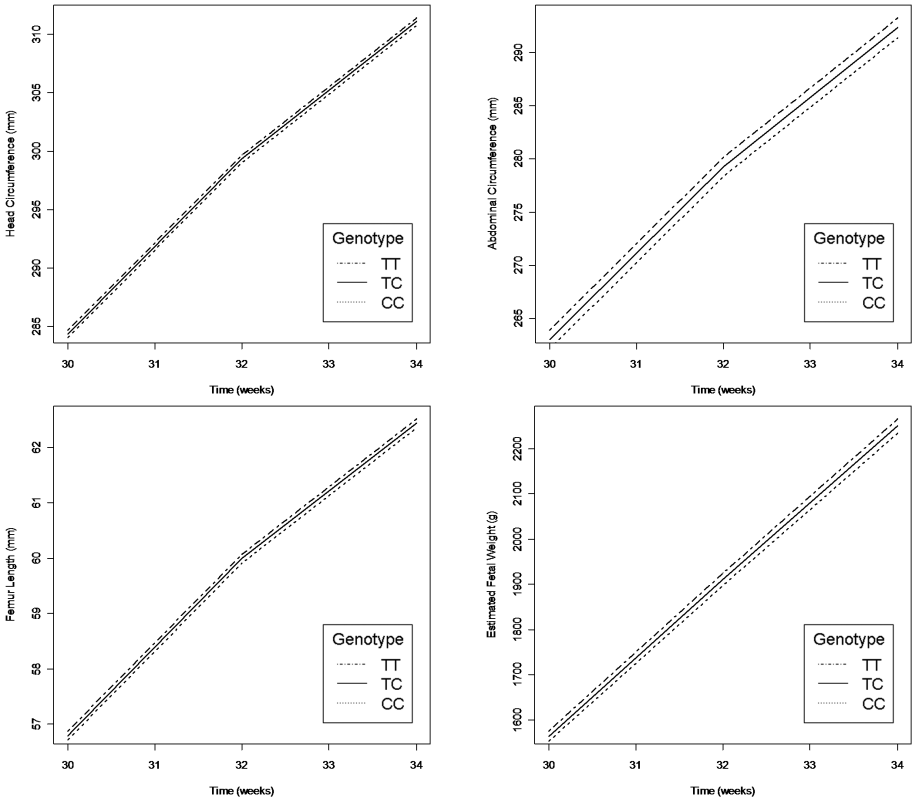
Figure 2a-d: Longitudinal association of rs900400 genotype with fetal growth in Raine.



fetal characteristics (HC p=0.048; AC p=0.0005; FL p=0.044; EFW p=0.0009), as compared to the T-allele in the Generation R Study (Figures 3 and 4). The findings were replicated for HC (p=0.02) and FL (p=0.09) in the Raine Study, but not for AC and EFW. At 32 weeks gestation in the Generation R Study, the CC genotype was associated with a head circumference that is 2 percentiles smaller and a femur length that is 1 percentile smaller than the TT genotype on clinical percentile charts.

Table 2 gives the cross-sectional analyses for the associations of rs900400 and rs9883204 with fetal growth characteristics in first, second and third trimester. Both the effect estimates for each cohort separately and the combined effect estimate are given. The C-allele of rs900400 was associated in second trimester with smaller fetal HC and FL (both p<0.05), and in third trimester with smaller HC, AC, FL and EFW (all p-values < 0.05). For each C-allele, the combined effect estimates for EFW were -1.66 (95% CI: -3.58, 0.26) grams and -19.2 (95% CI: -28.2, -10.3) grams in second and third trimester, respectively. The combined effect estimates for HC were -0.34 (95% CI: -0.65, -0.03) and -0.57 (95% CI: -1.02, -0.12) in second and third trimester, respectively. The C-allele of rs9883204, was not associated with fetal growth in first or second trimester. In third

Figure 3a-d: Longitudinal association of rs9883204 genotype with fetal growth in Generation R.



trimester, the C-allele of rs9883204 associated with AC (-1.18 (95% CI: -1.88, -0.47) mm), FL (-0.14 (95% CI: -0.26, -0.01) mm) and EFW (-18.5 (95% CI: -28.3, -8.6) grams), but not with HC (-0.43 (95% CI: -0.94, 0.07) mm).

Table 3 shows that at birth, the C-allele of rs900400 was associated with a reduction in all birth characteristics and placenta weight. For rs9883204, we found significant associations with decreased growth characteristics for the C-allele, except for HC. We found a strong association with lower placental weight amongst C-allele carriers of rs9883204 (-16.40 (95% CI: -23.70, -9.17) grams).

Discussion

In this study, we examined the associations between genetic variants near *CCNL1/LEKR1* (rs900400) and in *ADCY5* (rs9883204) with directly measured fetal growth characteristics in different trimesters in two independent prospective cohort studies from fetal life onwards. Rs900400 was already associated with growth restriction in second

Figure 4a-d: Longitudinal association of rs9883204 genotype with fetal growth in Raine.

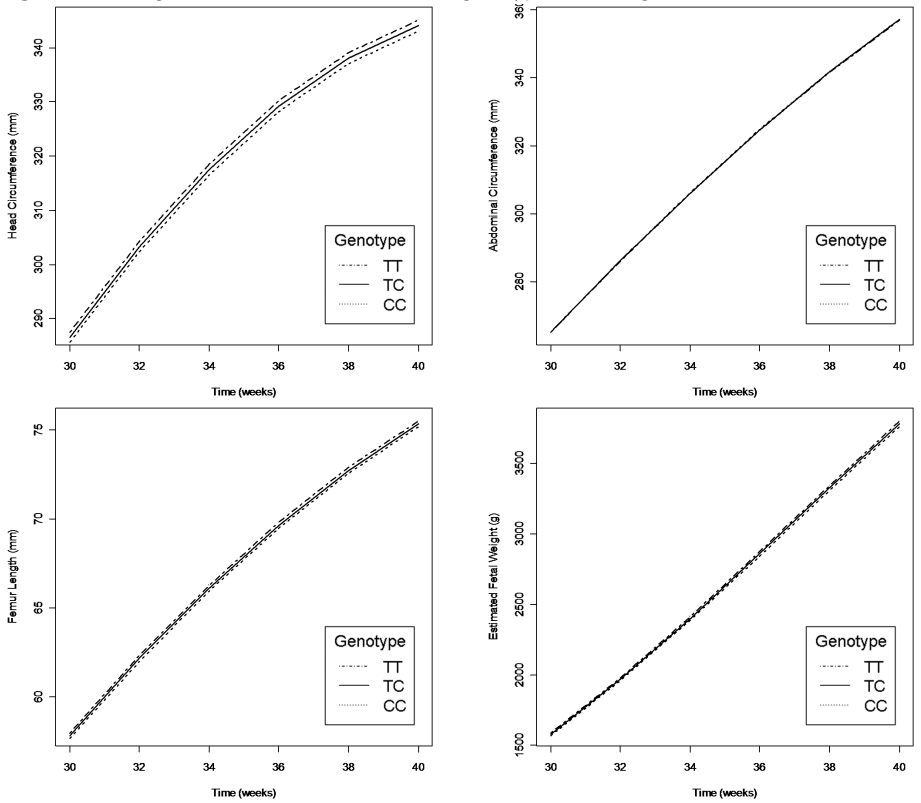


Table 2: Effect estimates for fetal growth characteristics for fetal rs900400 and rs9883204 genotype.

rs900400	HC (mm)	AC (mm)	FL (mm)	EFW (grams)
First trimester (12-16 weeks)				
Generation R (n=1,866)	-0.09 (-0.36, 0.18) p=0.53	-0.20 (-0.59, 0.19) p=0.31	-0.04 (-0.14, 0.06) p=0.46	0.04 (-0.98, 1.06) p=0.94
Second trimester (16-24 weeks)				
Generation R (n=2,559)	-0.25 (-0.58, 0.08) p=0.14	-0.29 (-0.74, 0.16) p=0.20	-0.10 (-0.19, 0.00) p=0.06	-2.05 (-4.40, 0.30) p=0.09
Raine (n=1,116)	-0.89 (-1.73, -0.04) **p=0.04**	-0.65 (-1.43, 0.13) p=0.11	-0.14 (-0.31, 0.03) p=0.14	-0.87 (-4.20, 2.46) p=0.61
Combined estimate	-0.34 (-0.65, -0.03) **p=0.03**	-0.38 (-0.77, 0.01) p=0.06	-0.11 (-0.20, -0.02) **p=0.01**	-1.66 (-3.58, 0.26) p=0.09
Third trimester (26-32 weeks)				
Generation R (n=2,582)	-0.49 (-0.96, -0.02) **p=0.046**	-1.10 (-1.81, -0.39) **p=0.002**	-0.20 (-0.32, -0.08) **p=0.001**	-19.8 (-29.8, -9.8) **p=1.17*10-4**
Raine (n=568)	-1.37 (-2.85, 0.12) p=0.07	-1.59 (-3.16, -0.02) **p=0.046**	-0.28 (-0.59, 0.03) p=0.07	-17.1 (-36.8, 2.6) p=0.09
Combined estimate	-0.57 (-1.02, -0.12) **p=0.01**	-1.18 (-1.83, -0.54) **p=3.16*10-4**	-0.21 (-0.32, -0.10) **p=1.87*10-4**	-19.2 (-28.2, -10.3) **p=2.38*10-5**

Table 2 (continued): Effect estimates for fetal growth characteristics for fetal rs900400 and rs9883204 genotype.

rs9883204	HC (mm)	AC (mm)	FL (mm)	EFW (grams)
First trimester (12-16 weeks)				
Generation R (n=1,866)	-0.19 (-0.48, 0.10) p=0.19	-0.35 (-0.78, 0.08) p=0.11	-0.05 (-0.15, 0.05) p=0.41	-0.18 (-1.30, 0.94) p=0.76
Second trimester (16-24 weeks)				
Generation R (n=2,559)	-0.13 (-0.48, 0.22) p=0.49	-0.15 (-0.64, 0.34) p=0.54	-0.08 (-0.19, 0.04) p=0.15	-2.00 (-4.57, 0.57) p=0.13
Raine (n=1,116)	-0.09 (-1.03, 0.85) p=0.86	-0.08 (-0.96, 0.80) p=0.85	0.03 (-0.17, 0.23) p=0.79	0.70 (-3.02, 4.42) p=0.71
Combined estimate	-0.12 (-0.46, 0.21) p=0.45	-0.13 (-0.56, 0.30) p=0.54	-0.05 (-0.15, 0.05) p=0.32	-1.13 (-3.24, 0.98) p=0.29
Third trimester (26-32 weeks)				
Generation R (n=2,582)	-0.30 (-0.83, 0.23) p=0.26	-1.36 (-2.12, -0.60) **p=4.9*10-4**	-0.13 (-0.27, 0.00) p=0.06	-20.6 (-31.6, -9.6) **p=2.4*10-4**
Raine (n=568)	-1.73 (-3.38, -0.08) **p=0.04**	-0.18 (-1.96, 1.60) p=0.84	-0.18 (-0.53, 0.17) p=0.32	-9.3 (-32.0, 13.4) p=0.42
Combined estimate	-0.43 (-0.94, 0.07) p=0.09	-1.18 (-1.88, -0.47) **p=0.001**	-0.14 (-0.26, -0.01) **p=0.04**	-18.5 (-28.3, -8.6) **p=2.5*10-4**

All effects (standard error) are given for the C-allele (minor allele for rs900400 and major allele for rs9883204).
HC: head circumference; AC: abdominal circumference; FL: femur length; EFW: estimated fetal weight
Effects are estimated using the following model:
Growth parameter ~ SNP + sex + gestational age

trimester, though this association was only significant for head circumference and femur length. In third trimester, this variant was associated with all fetal growth characteristics. Rs9883204 was not associated with fetal growth until third trimester. In third trimester, this variant was associated with smaller fetal growth characteristics except head circumference. Furthermore, both loci were related to placental weight, but the strongest effect was observed for *ADCY5*.

Birth weight is merely a summary or end-point of fetal growth. Different fetal growth patterns can result in the same birth weight.[20] Adverse fetal growth in different periods during the pregnancy can have different effects on adult metabolic phenotypes.[21] We demonstrated that the variant near *CCNL1/LEKR1* already starts to affect fetal growth from second trimester onwards and that both loci are associated with third trimester fetal growth characteristics. Birth weight is determined by skeletal and non-skeletal body parts including length and weight measures. We have previously demonstrated that rs900400 influenced birth weight through a lower ponderal index.[1] However, the

Table 3: Effect estimates on birth characteristics for rs900400 and rs9883204 genotype.

rs900400	**Head circumference (mm)**	**Length (mm)**	**Weight (grams)**	**Ponderal index (kg/m³)**	**Placental weight (grams)**
Generation R (n=2,661)	-0.09 (-0.19, 0.01) p=0.10	-0.28 (-0.42, -0.14) **p=1.1*10-5**	-62.70 (-86.61, -38.79) **p=3.0*10-7**	-0.04 (-0.24, 0.16) p=0.71	-7.32 (-16.04, 1.40) p=0.10
Raine (n=1,197)	-0.07 (-0.17, 0.03) p=0.16	-0.09 (-0.24, 0.07) p=0.27	-56.77 (-91.76, -21.78) **p=0.002**	-0.35 (-0.57, -0.13) **p=0.002**	-6.27 (-16.21, 3.67) p=0.22
Combined estimate	-0.08 (-0.15, -0.01) **p=0.02**	-0.20 (-0.30, -0.09) **p=1.76*10-4**	-60.8 (-80.6, -41.1) **p=1.56*10-9**	-0.18 (-0.33, -0.04) **p=0.01**	-6.86 (-13.40, -0.31) **p=0.04**
rs9883204	**Head circumference (mm)**	**Length (mm)**	**Birth weight (grams)**	**Ponderal index (kg/m³)**	**Placental weight (grams)**
Generation R (n=2,661)	0.03 (-0.09, 0.15) p=0.65	-0.12 (-0.26, 0.02) p=0.09	-42.04 (-68.36, -15.72) **p=0.002**	-0.09 (-0.31, 0.13) p=0.39	-15.44 (-24.93, -5.95) **p=0.001**
Raine (n=1,197)	-0.12 (-0.24, 0.00) p=**0.04**	-0.12 (-0.30, 0.06) p=0.18	-61.70 (-101.29, -22.11) **p=0.002**	-0.38 (-0.63, -0.13) **p=0.003**	-17.80 (-29.05, -6.55) **p=0.002**
Combined estimate	-0.05 (-0.13, 0.04) p=0.29	-0.12 (-0.23, -0.01) **p=0.03**	-48.1 (-70.0, -26.1) **p=1.72*10-5**	-0.21 (-0.38, -0.05) **p=0.01**	-16.40 (-23.70, -9.17) **p=9.09*10-6**

All effects (standard error) are given for the C-allele (minor allele for rs900400 and major allele for rs9883204). Effects are estimated using the following model:
Growth parameter ~ SNP + sex + gestational age

present study shows associations of rs900400 with all fetal growth characteristics, both in the cross-sectional and longitudinal analyses, which is suggestive of symmetric growth restriction in carriers of the C-allele. In the genome-wide association study on birth weight, rs9883204 appeared to affect birth weight through birth length.[1] Femur length in utero is correlated to total body length.[22] We found associations of rs9883204 with both femur length in third trimester and birth length. Interestingly, both in third trimester and at birth, rs9883204 was associated with all fetal growth characteristics, except fetal head circumference, which could indicate asymmetric growth restriction. These results were not consistent with the longitudinal results, where we found an association with decreased head circumference growth in both studies. However, in the Generation R Study the effect size for in the longitudinal analyses for abdominal circumference was larger than head circumference, which still would be indicative of asymmetric growth. Both loci were related to placental weight, but the strongest effect was observed for *ADCY5*. This latter association could be an indication of an influence of the gene on placentation or placental growth. This would fit well with the knowledge

that decreased placental function causes redistribution of the fetal circulation in favor of the cerebral blood flow.[23] However, in the Rain Study there was no association between rs9883204 and placental function measured as uterine artery blood flow (data not shown). For both the rs900400 and rs9883204, we have previously demonstrated that maternal genotype was not associated with birth weight.[1] Also, in the present study, maternal *ADCY5* genotype was not associated with placental weight, indicating that the fetal genotype is most likely affecting the placental growth and thus influencing birth weight (data not shown).

Hattersley and Tooke proposed that common genetic variants that are associated with decreased fetal growth might also cause type 2 diabetes in adulthood.[4] This hypothesis is known as the fetal insulin hypothesis. This hypothesis is supported by a recent meta-analysis showing consistent and robust association between low birth weight and type 2 diabetes.[24] The rs9883204 in the *ADCY5* gene is associated with both type 2 diabetes in adulthood and birth weight, giving robust evidence for the fetal insulin hypothesis.[1,2] It could be hypothesized that the asymmetric growth restriction due to reduced insulin secretion associated with variations in *ADCY5* may lead to adaptations which have metabolic consequences in adulthood. Follow-up studies have suggested that both low birth weight, low birth length, low ponderal index and low placental weight are all associated with the risk of type 2 diabetes.[25]

Some methodological issues need to be considered. First, in both studies gestational age was based on crown-rump length measured in early pregnancy in most subjects. Determining gestational age based on the first day of the last menstrual period is often subject to recall bias and therefore less accurate. With the crown-rump length method the assumption is that growth is uniform in the first trimester.[26] However, we have previously demonstrated that first-trimester crown-rump length can be influenced by a variety of factors.[27] First-trimester crown-rump length is also correlated with fetal growth characteristics in second and third trimester.[27] Thus, using first-trimester crown-rump length as method for pregnancy dating may lead to underestimation of the associations, especially in the first half of pregnancy. Also, though we have shown that ultrasounds measurements in early pregnancy are accurate and highly reproducible, the variance in these measurements may be too small to detect small genetic effects.[28] For example, the standard deviation of the femur length measurement in early pregnancy is 5 mm. An effect size of 0.1 standard deviations equates to 0.5 mm, which is much smaller than the measurement error. Finally, our study used data from two unique datasets with directly assessed repeated fetal growth measurements. Nonetheless, our sample size is limited, considering that most genetic association studies are performed in over 10,000 subjects.

In conclusion, we demonstrated that two common genetic variants that have been shown to be associated with birth weight affect fetal growth in different ways. Rs900400 affects fetal growth from early pregnancy onwards, and our results are suggestive for a

symmetric growth restriction. Rs9883204 is associated with fetal growth restriction from third trimester onwards and showed evidence of asymmetric growth restriction, with no effect on head circumference. Furthermore, the genetic variant of *ADCY5* was strongly associated with placental weight.

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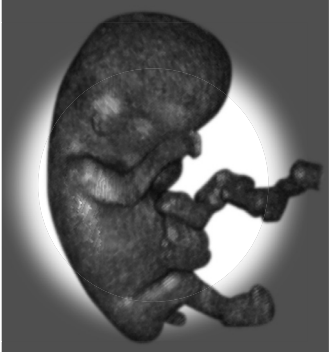

CHAPTER 5

General discussion

Introduction

Over the past decade epidemiological studies have demonstrated an inverse relationship between birth weight and adult diseases, such as type 2 diabetes (T2D).[1] These relationships have been found to be quite robust and cannot be explained by confounding factors such as prematurity, maternal smoking or socio-economic class.[2] However, in a recent meta-analysis the effect size was shown to be not as large as originally estimated.[2]

Two hypotheses have been developed with proposed pathways for these associations, namely the 'developmental origins of health and disease (DOHaD) hypothesis' and the 'fetal insulin hypothesis'. The proposed causal pathway of the DOHaD hypothesis is a suboptimal fetal environment, which leads to metabolic adaptations in the fetus to increase short-term survival rate by selecting an appropriate growth trajectory. This adaptation is also known as fetal programming.[3] Though this programming might lead to an increased survival rate in utero, the developmental adaptations can have long-lasting effects on the risks of common diseases in adulthood.[3] Alternatively, the proposed pathway of the 'fetal insulin hypothesis' is that common genetic variants related to insulin sensitivity or secretion explain, at least in part, the associations between poor fetal growth and type 2 diabetes in adulthood.[4] This pathway focuses primarily on the risk of diabetes and not on other metabolic and cardiovascular disease. Since insulin is the most important fetal growth factor, these genetic variants could cause both low insulin-mediated fetal growth and low insulin secretion or resistance in adulthood.[4]

Birth weight is often used as the main measure of fetal growth since it is readily available in obstetrics records. There are two main problems with using birth weight as a proxy for fetal growth when studying the associations between fetal growth and adult diseases. Firstly, it is important to realize that birth weight is a cross-sectional measurement, thus merely an end-point of fetal growth and the starting-point of postnatal growth. Different growth trajectories may lead to the same birth weight and children with the same birth weight can have different postnatal growth trajectories.[5] Furthermore, adverse environmental and/or genetic influences may have different effects during various periods of pregnancy. Therefore, the study of fetal growth trajectories using multiple measurements may be more informative than birth weight as a single measurement. Moreover, when exploring the relationship between fetal growth and disease in adulthood, it is important to distinguish low birth weight (< 2,500 grams) from small size for gestational age (< -2 standard deviation score). Secondly, fetal adaptations and decreased growth during different periods may have different postnatal consequences.[3] In the Dutch Famine study, maternal undernutrition in the first trimester of pregnancy was associated with increased rates of coronary heart disease and increased lipid levels in adulthood, while undernutrition in late pregnancy was related to glucose intolerance.[3]

Thus, when studying the fetal origins of adult disease, it is also important to take into account during which period of pregnancy the fetal adaption occurred.

The aim of the studies presented in this thesis was to explore both pathways mentioned above using directly measured fetal growth. In this chapter, we give an overview of the most important epidemiological studies regarding the associations between fetal growth and diseases in adulthood, including what the current studies of this thesis add to this field of research. Furthermore, we consider methodological issues and give suggestions for further research.

Main findings

Early growth and body composition

Since the initial observation that birth weight is related to metabolic diseases in adulthood, a lot of epidemiological research has examined possible pathways which could explain such associations. We have demonstrated in a population-based cohort that maternal characteristics and adverse lifestyle habits are associated with first trimester fetal growth restriction.[6] Importantly, first trimester growth restriction was associated with a 2- to 3-fold increased risk of being born premature and small-for-gestational age.[6] Moreover, first trimester growth restriction was associated with an increased growth rate postnatally, a known risk factor for cardiovascular and metabolic disease in adulthood.[6] We have therefore hypothesized that adverse lifestyle habits, such as smoking, could already have detrimental effects on the fetus before the mother even knows she is pregnant. Though longer follow-up studies are scarce, smoking during pregnancy has been shown to increase blood pressure and the risk of childhood obesity in the offspring.[7-10] Studies have demonstrated that blood pressure and body composition track during life and therefore these adaptations in early growth could have long lasting metabolic effects.[11-13]

The relationship between birth weight and obesity is complicated. Most studies indicate that the association is most likely to be J-shaped, where both low birth weight and high birth weight are associated with obesity.[14] In this thesis, we showed evidence that would fit well with this J-shaped association. We demonstrated that children growing in the highest quintile of estimated fetal weight during the first half of pregnancy have a higher peak weight velocity during the first month after birth. On the other hand, children with a birth weight in the lowest quintile also have an increased peak weight velocity, a phenomenon which is associated with an increased risk of obesity and higher blood pressure in adulthood.[13] We also demonstrated that an increased growth rate during the first two years of life, also known as catch-up growth, was positively associated

with several measures of abdominal fat mass at the age of 2 years.[15] Catch-up growth until the age of 2 years has been extensively studied and is known to be associated with adverse metabolic phenotypes in childhood and adulthood.[16-18] However, children who were born small-for-gestational-age and had rapid weight gain as early as during the first 3 months of life were at increased risk of cardiovascular disease and type 2 diabetes.[19] Thus, it seems that rapid weight gain in the first months immediately after birth may be of greater importance than catch-up growth in the first two years.[20]

Finally, growth from fetal life onwards is known to be highly heritable. Heritability is the proportion of variability of a phenotype which can be explained by shared genes and environment. Total adult body height is considered to be a highly heritable trait, with an estimated heritability of about 90%,[21] while the heritability of weight and body mass index is considered to be lower with estimates ranging from 16% to 85%.[22] In this thesis, we estimated the heritability of body size from second trimester until the postnatal age of 36 months. During this period, the heritability for height and weight increased from 12.6% to 63.4% and from 16.6% to 42.3%, respectively. Longer follow-up studies are necessary to examine how the heritability develops in later childhood and puberty. Furthermore, studies targeting specific genetic pathways may clarify what genes contribute to the heritability of growth during fetal and early postnatal life.

Genetics of early growth

Insulin is the most important fetal growth factor. Genetic polymorphisms may decrease insulin secretion or sensitivity and lead to decreased fetal growth and increased risk of type 2 diabetes in adulthood.[4] Another explanation for a genetic link between early growth and metabolic phenotype in adulthood is that some genetic polymorphisms might have different effects during various periods of life.

Before genome-wide association studies were available, genetic association studies focused on the effects of candidate genes. A number of these candidates were the glucocorticoid receptor gene,[23] the insulin gene,[24-28] genes encoding for insulin-like growth factors and their binding proteins,[29,30] and genes involved in the renin-angiotensin-aldosterone-system.[31] Frequently, a positive association was found between a genetic polymorphism, either from maternal or fetal genotype, and fetal growth/birth weight and adult metabolic phenotype.[23-31] These publications were subsequently followed by several other independent studies that were unable to replicate these results.[32-35] Recent developments in genetic epidemiology have decreased the number of false-positive findings dramatically. First of all, genome-wide association studies allow investigators to study genetic association in a hypothesis-free manner. Instead of analyzing one or a number of genetic variants, associations for about 2.5 million SNPs can be explored at once. Secondly, the sample size of genetics association studies has increased from a

few hundred individuals per study to over 10,000 subjects per study. And finally, most genetic association studies are required to report replication of their findings in an independent cohort.

The identification of *FTO* as an obesity gene was one of the first successes of the genome-wide association studies on obesity.[36] The biological mechanisms behind this association are yet to be fully determined, but evidence from both population based analyses and functional investigations have suggested that this locus is likely to be involved in the hypothalamic regulation of appetite or energy expenditure and metabolic rate.[37-41] Also, the association between BMI and variation at *FTO* appeared age-dependent with the heritability for BMI increasing with age, and the proportion of variance in BMI explained by shared environment diminishing over the same time period.[42] In this thesis, we present two studies regarding *FTO* and early growth. Firstly, we found no association between this locus and body composition measured by dual energy x-ray absorptiometry (DXA) at the age of 6 months.[43] In a large cohort of over 5,000 children aged 9 years, the risk allele of *FTO* gene was associated with an increased total fat mass measured by DXA.[36] Another smaller study of 234 newborns showed a similar increase in fat mass by means of DXA in the variant carriers of this *FTO* polymorphism as early as two weeks after birth.[44] We were not able to replicate these results at the age of 6 months, indicating that the effect of *FTO* most likely is age-dependent. Secondly, we performed a large, longitudinal, investigation into the association of a variant of *FTO* with BMI. The results not only confirmed the increasing magnitude of associations between this variant and adiposity from the end of infancy through to childhood, but also suggested an inverse association at very early ages (< 2.5 years). Beyond the age of 5.5 years, the anticipated patterns of association between *FTO* and BMI gradually emerged. The age-dependent nature of association between *FTO* and BMI provides important information about longitudinal gene effects and specifically about the role of *FTO* in adiposity.

The gene with the largest effect on type 2 diabetes is *TCF7L2*.[45] This *TCF7L2* polymorphism has been suggested to reduce proinsulin to insulin conversion,[46] though the exact mechanism has not been elucidated yet. Carriers of two risk alleles have about a 2-fold increased risk of developing type 2 diabetes.[45] This gene might also affect early growth. Freathy *et al*. demonstrated an effect on birth weight of a polymorphism in this gene, but the authors stated that this most likely was due to increased glucose levels in mothers associated with maternal genotype.[47] We demonstrated in two studies that the fetal genotype of this polymorphism indeed had no effect on fetal growth.[48] Another polymorphism that was shown to be associated with type 2 diabetes was *PPARγ-2* Pro12Ala.[45] The Pro12Pro genotype was associated with an increased risk of type 2 diabetes.[45] On the other hand, a large meta-analysis had indicated that the Ala12 allele was associated with obesity.[49] This finding has however not been confirmed by recent genome-wide association studies on body mass index in adulthood.[50] In this thesis, we present a

study that demonstrated an increased growth rate amongst carriers of the Ala12 allele between birth and the age of 2 years.[51] The growth rate is only accelerated in children who were breastfed less than four months.[51] In children who were breastfed longer than four months there was no association between this polymorphism and growth.[51] These results were replicated in an independent study, where the authors found an increased body mass index in adolescence in Ala12 carriers who were not breastfed.[52]

Finally, in this thesis we present the first genome-wide association study on birth weight.[53] We identified two loci to be associated with birth weight, one near *CCNL1/LEKR1* and the other in *ADCY5*.[53] Correlated SNPs in *ADCY5* were recently implicated in regulation of glucose levels and type 2 diabetes susceptibility,[54] providing evidence that the well described association between lower birth weight and subsequent type 2 diabetes[55,56] has a genetic component, distinct from the proposed role of programming by maternal nutrition. Also other genetic polymorphisms, namely in *CDKAL1* and *HHEX*, that have been identified to be associated with type 2 diabetes have recently been shown to also affect birth weight.[57,58] We also demonstrated that these genetic polymorphisms affect fetal growth in different ways. The variant near *CCNL1/LEKR1* affected fetal growth from early pregnancy onwards, and our results are suggestive for a symmetric growth restriction. In contrast, the variant in *ADCY5* was associated with fetal growth restriction from third trimester onwards and showed evidence of asymmetric growth restriction, with no effect on head circumference. Furthermore, the genetic variant of *ADCY5* was strongly associated with placental weight.

In conclusion, several genetic polymorphisms related to metabolic phenotype in adulthood have been identified to affect early growth. These studies were performed in large studies or a consortium of multiple studies, thus limiting the probability of false-positive findings.

Methodological considerations

Selection bias

Most of the studies in this thesis were carried out in the Generation R Study, a population based prospective cohort study from early fetal life onwards. The Generation R Study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood.[59,60] Off all children eligible at birth within the study area, 61% participated in the Generation R Study.[59-61] Selection bias may occur when participation to the study is related to either the determinant or the outcome, or both. In the Generation R Study, non-response is not likely to be at random.[59-61] For example, mothers with a Dutch ethnicity and with a university degree were more

likely to participate than were mothers from ethnic minorities or mothers with only a high-school degree.[59-61] In this example, selection could be problematic when the selection mechanisms are associated with both the educational level of mothers and fetal growth, and these associations differ between the selected population and the eligible population.[62] Importantly, the selection bias towards a more homogeneous population may diminish variability of the outcome and decrease the power of the study.

Selection bias in our studies might also arise from selective loss to follow-up. In most of the studies regarding postnatal growth, children who were included in the follow-up had a higher birth weight and gestational age at birth than those who were lost to follow-up. Children born with a low birth weight or born preterm have a different postnatal growth pattern, such as a higher rate of catch-up growth, than children born with a normal birth weight and gestational age. Thus, this selective loss to follow-up towards a more homogeneous and healthy population may have biased our effect estimates.

Finally, regarding the genetic association studies in this thesis, all DNA samples from the children were isolated from cord blood. Cord blood for DNA isolation was available in 59% of all live born participating children. Children with genetic data available had a lower birth weight and were born at a lower gestational age. A number of studies in this thesis examined the association between genetic variants and birth weight.[48,51,53] Since the selection of the children is related to the outcome, i.e. birth weight, this may bias our results. Population stratification based on selection is unlikely since all the genotypes were in Hardy-Weinberg equilibrium. Furthermore, in the genome-wide association study we restricted our population to children born after 37 weeks of gestation, which most likely limited the selection bias.[53]

Information bias

In the genetic association studies in this thesis, misclassification or information bias can occur on both the determinant, i.e. the gene, and the outcome. Since DNA was collected at birth from cord blood, it is possible that some samples were mixed with maternal samples. However, the prevalence of this type of mismatch was low. We cross-referenced the sex of the offspring based on their genome with the information obtained from the midwives and the sex-mismatch rate was low (<0.5%). This misclassification can be considered at random and would lead to an underestimation of effect estimates. More important, however, is misclassification of the outcome, i.e. growth outcomes. For postnatal growth, all growth meaurements were standardized and therefore it is unlikely that measurement error was large. All prenatal growth models were adjusted for the gestational age at the time of the ultrasound measurement. Similarly, models for birth outcomes took gestational age at birth into account. Gestational age was, as routine obstetric protocols prescribe, mostly based on crown-rump length measured in

early pregnancy.[63] Basing gestational age on crown-rump length assumes that growth is uniform during this period.[63] However, in this thesis we argue that crown-rump length in first trimester can be affected by maternal characteristics and lifestyle habits.[6] Furthermore, crown-rump length in first trimester was highly correlated to growth in second and third trimester.[6] Since the misclassification of gestational age is expected to be random, it would bias the effect estimates towards the null. The misclassification of gestational age also applies to the non-genetic studies in this thesis. Moreover, these studies used questionnaire data to gather a large amount of information on determinants and covariates. Reporting of lifestyle habits by questionnaires might lead to non-random misclassification. For example, mothers might overestimate the number of months that they breastfed, while they underreport the number of cigarettes they smoked during pregnancy.[64] The effect estimates of breastfeeding duration on growth would most likely be underestimated, while the effect of smoking would then be overestimated. To overcome this form of information bias, studies will need to focus on biomarkers and metabolites that can serve as intermediates between the exposure and outcome, though finding appropriate biomarkers which are superior to questionnaire data has been proven to be difficult.[65]

Confounding

A confounder is a factor, associated with both the determinant and the outcome, which leads to a biased effect estimate. For the genetic association studies in this thesis, all models were unadjusted, since population genotype distribution was assumed to be unrelated to covariates such as maternal age, pre-pregnancy body mass index or parity.[66] Confounding in these studies therefore can be assumed to be minimal. For non-genetic association studies in this thesis we may have missed potential confounders or did not measure potential confounders appropriately, also known as residual confounding. For example, in the study of risk factor and outcomes associated with early growth restriction we did not include information on maternal diet. It cannot be excluded that maternal diet is both associated to a risk factor, such as maternal smoking, and to early growth, and that therefore our effect estimates could be biased. In observational studies, residual confounding is always a limitation, which might be addressed by performing randomized trials.

Implications of new genetic findings

In this thesis we describe several genes that are associated with early growth. In recent years, there has been tremendous progress in the identification of new genes involved

in complex traits and diseases.[67] Genome-wide scans have contributed greatly to these efforts.[67] The great promise of these genetic discoveries was that one day they would lead to 'personalized medicine', which would allow medical decision regarding the prevention and treatment of disease to be tailored to the genetic profile of an individual.[68] For example, if a physician knew based on a mother's personal genetic profile that the fetus was at an increased risk of growth restriction, the physician might decide to increase the frequency of ultrasound examinations.

In the last few years commercial enterprises, such as deCODEme, 23andMe and Navigenics, have started to offer 'personal genome scans', with the primary goal of allowing clients to obtain insight into their risk of a number of diseases. These commercial tests are usually not part of the routine health-care system, but are offered via the internet. Most of the diseases that are tested are multifactorial or complex diseases, such type 2 diabetes, coronary heart disease and obesity. Lately, new genetic discoveries have been receiving a lot of attention both in medical and non-medical media. Therefore, patients will increasingly have questions about how genes influence disease. Furthermore, people who purchase personal genome scans will not know how to interpret their results.

Since most SNPs only have a small effect and since usually a variety of genes are involved, prediction models for complex diseases are routinely not based on a single SNP but on risk scores of multiple SNPs, also called a *genetic profile*.[69] These risk scores are often associated with the risk of disease,[70] but this association does not mean that these risk scores are useful for disease prediction. Actually, the predictive value of genetic profiles has been shown to be very limited and only marginally improves the prediction of disease based on non-genetic risk factors, such as obesity and smoking.[69] Furthermore, risk scores based on personal genetic profiles usually do not take the age and sex of the client into account. For example, for the risk of cardiovascular disease it really makes a difference whether one is predicting the risk of disease for a 20-year old female or a 55-year old male. Also, for many of the risk genes there are no known (pharmaceutical) interventions available. In addition, some interventions (for example, eat healthy or stop smoking) should ideally be recommended to everybody, regardless of the genetic profile. Finally, some tests offer a screening for heritable monogenetic diseases, such as mutation in *BRCA1* en *BRCA2* (breast cancer). However, these tests only screen a couple of the several thousands of known mutations in these genes and could therefore wrongly lead to a feeling of comfort.

Despite the limited predictive value, the results of the genome-wide association studies have been very promising. Not only have there been many new discoveries, but also new pathogenic mechanisms have been identified. In this thesis we describe a genetic link between low birth weight and type 2 diabetes in adulthood.[71,72] Nonetheless, these findings are not clinically relevant, since the SNPs usually have a relatively small effect.[72] For example, in the case of type 2 diabetes, carriers of two of the*TCF7L2* risk alleles have

only about a 2-fold increased risk of developing type 2 diabetes. This SNP in the *TCF7L2* gene was identified to have the strongest effect.[71] Most other SNPs had a relative risk lower than 1.3.[71] In comparison, the risk of type 2 diabetes is about eight times higher when a person has a body mass index (BMI) above 25 kg/m2 and a hundred times higher with a BMI above 35 kg/m^2 compared to an individual with a BMI below 22 kg/m^2.[73] In the near future we undoubtedly can expect many new SNPs to be indentified for these diseases. These discoveries will also change the risk calculation based on personal genetic profiles.[74] However, it is unlikely that these new SNPs will be very prevalent in the normal population, i.e. more than 1%, and/or lead to large increase in risk.[75]

In conclusion, the personal genetic profiles that are available through commercial enterprises are of no clinical use for the moment. The test results should generally be seen as inconclusive. A physician should be able to explain to the patient that the risk increase based on personal genetic profile is marginal in most cases (except for Huntington's disease and some heritable forms of cancer) compared to the risk increase due to conventional factors. These non-genetic factors are usually not taken into account when these companies make risk predictions. Physicians are capable of making a better prediction of disease based on their clinical insight and the presence of known risk factors (family history, smoking, obesity, etc.) than is currently possible based on personal genetic profiles. In conclusion, physicians should be careful about giving advice based on the results of such tests and should remain focused on the conventional risk factors.

Future research

Most epidemiological research studying various pathways for the association between low birth weight and disease in adulthood, including the studies presented in this thesis, focuses either on fetal adaptive mechanisms to environmental factors or genetic factors. However, the greatest progress in this field of research will be made by combining these two pathways. In the coming years, research will be focused on epigenetics, metabolites and biomarkers, and genome-wide association studies on gene-environment interactions.

Epigenetics

Epigenetic research is a rapidly growing field that focuses on heritable changes in gene expression that are not caused by DNA sequence modifications.[76] The addition or removal of methyl groups to the DNA, known is methylation, is the most known form of epigenetic modification. Epigenetic changes in the *IGF2-H19* region on chromosome 11p15 are thought to have important effects on growth.[77,78] In Silver-Russell syndrome,

a syndrome associated with pre- and postnatal growth retardation, the *IGF2-H19* region is hypomethylated, which subsequently leads to an underexpression of insulin like growth factor-2 (IGF2).[79] On the other hand, this same region is hypermethylated (leading to overexpression of IGF2) in Beckwith-Wiedemann, a syndrome associated with overgrowth.[80] Recently, lower DNA methylation of this region was found in subjects who were exposed to undernutrition during the Dutch Famine.[81] The authors of the study hypothesized that these changes in DNA methylation patterns could cause reduced growth in early life and diseases, such as type 2 diabetes and cardiovascular disease.[81] One of the most important sources of methyl groups in our daily diet is folic acid.[82] In this thesis, we demonstrated that non-use of folic acid intake around conception was associated was decreased growth as early as the first ten weeks of pregnancy.[6] Adequate folic acid intake during pregnancy has also been shown to reduce the risk of prematurity and low birth weight.[83] Thus, future research will need to focus on how folic acid affects methylation patterns (not only of the *IGF2-H19* region but across the entire human genome) and how these methylation changes might play a role in the association between early growth and disease in adulthood.

Metabolites and biomarkers

A recent genome-wide association study identified several new loci to be associated with metabolites, such as amino acids and phospholipids.[84,85] This study had a novel design, since it was hypothesis-free both on the genetic side and the metabolite side. Aside from the newly identified loci, the most important finding was that genes could explain up to 36% of the variance in metabolite concentrations.[84,85] In comparison, the two genome-wide hits on birth weight reported in this thesis had an explained variance of 1-2% at most.[53] Furthermore, a number of the identified metabolites had previously been reported to be associated with clinically relevant outcomes, thus indicating that the metabolites are serving as an intermediate between the gene and the disease.[84,85]

This study was performed in adults, but the same design could be used to explore the association between fetal growth and disease. Future research on these metabolites and other biomarkers (for example early markers of abnormal placentation, such as pregnancy-associated plasma protein A and free human chorionic gonadotropin-β) in early life may give us a better understanding as to which genes influence certain fundamental metabolic functions, and how these metabolites and biomarkers subsequently may affect growth in early life and the risk of disease in adulthood.

Genome-wide association studies on gene-environment interactions

In this thesis, we describe an interaction between the Pro12Ala polymorphism in *PPARγ-2* and breastfeeding duration on early growth rates.[51] These results were replicated when a similar interaction was found between this polymorphism and breastfeeding duration on obesity in adolescents.[51] Interestingly, a recent large meta-analysis of genome-wide association studies on adult obesity did not find the Pro12Ala variant to be associated with body mass index.[50] A possible explanation is that this polymorphism is only active in certain environmental situations. The genome-wide association studies did not take environmental factors into account.[50] Another example is smoking and birth weight. In this thesis, we describe a genome-wide association study on birth weight resulting in two genome-wide hits.[53] It could be hypothesized that stratification in smokers and non-smokers would identify genes that are associated with birth weight in a smoking or non-smoking environment, respectively.

Before such studies are successful, we need to overcome the heterogeneity of the environmental factor. Most studies are not large enough both to identify genome-wide significant hits on their own and also to serve as good replication studies. Therefore, collaborative efforts are often necessary. When combining the results of these studies, one has to take into account that information on smoking or breastfeeding duration was obtained in different ways. Furthermore, enrolment of the studies might have occurred several decades apart from each other which increases the heterogeneity even further, e.g. the prevalence of maternal smoking during pregnancy was much higher in the 50s than in the 90s.[53] Future methodological research will therefore also need to focus on how to overcome these differences in environmental phenotype.

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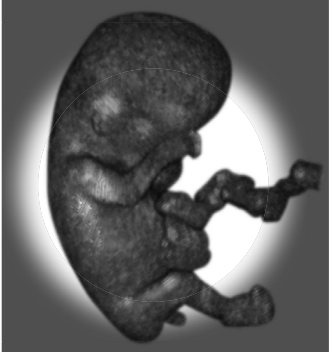

CHAPTER 6

Summary / Samenvatting

Summary

Epidemiological studies have demonstrated an inverse relationship between birth weight and the risk of type 2 diabetes. Recently, this relationship has been found to be quite robust, though the effect size might not be as large as originally estimated. There are two hypotheses that propose causal pathways underlying the association between low birth weight and metabolic phenotype: the 'developmental origins of health and disease hypothesis (DOHaD hypothesis) and the 'fetal insulin hypothesis'. The main idea behind the DOHaD hypothesis is that a suboptimal fetal environment leads to fetal undernutrition. This undernutrition subsequently causes developmental adaptations that permanently alter fetal growth, physiology and metabolism, also referred to as fetal programming. Though this programming might lead to an increased survival rate in early life, the developmental adaptations can have long-lasting effects on disease. Alternatively, the 'fetal insulin hypothesis' proposes that common genetic variants related to type 2 diabetes might also explain, at least in part, the association between poor fetal growth and metabolic phenotype. Since insulin is the most important fetal growth factor, these genetic variants could cause both low insulin-mediated fetal growth and low insulin secretion in adulthood.

Birth weight is merely the end-point of fetal growth and the starting point of postnatal growth. Historically, most epidemiological studies that focused on this association used birth weight, a cross-sectional observation, as a measure of early growth. However, fetal adaptations in growth and metabolism might already occur in the first weeks of pregnancy. Longitudinal growth studies have shown that growth rate might also play a role in metabolic changes leading to disease in adulthood. Using directly measured fetal growth characteristics from early pregnancy onwards as measure of early development may give greater insight into the timing of changes in early life that lead to the association between low birth weight and later disease.

There were three main objectives of this thesis: 1) To investigate what factors influence early growth and to study how early growth influences body composition in childhood, 2) to examine the associations of genetic variants known to be associated with obesity and type 2 diabetes with growth and body composition in early childhood and adolescence, and 3) to identify, by means of genome-wide association studies, new genetic variants that are associated with fetal growth. For most studies in this thesis, we used data from the Generation R Study, a population-based prospective cohort study. This study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood. Several of the genetic association studies were in close collaboration with the Early Growth Genetics (EGG) consortium.

In **chapter 2**, we studied the relationship between pre- and postnatal growth and body composition and factors that influence this relationship. In **chapter 2.1**, we examined what risk factors and outcomes are associated with first-trimester growth restriction. For this, we evaluated the associations of maternal physical characteristics and lifestyle habits with first-trimester fetal crown to rump length in mothers with a known and reliable first day of last menstrual period and regular menstrual cycle. We found that maternal age, lower diastolic blood pressure and lower hematocrit levels were positively associated with first-trimester fetal crown to rump length. Non-smoking and optimal use of folic acid supplements also were associated with a larger fetal crown rump length. Compared to normal first-trimester fetal growth, first-trimester growth restriction was associated with 2-to-3 fold increased risks of preterm birth, low birth weight and small size for gestational age at birth. First trimester crown to rump length was also negatively associated with postnatal growth acceleration until the age of 2 years, a known risk factor for type 2 diabetes and cardiovascular disease in adulthood. We concluded that maternal physical characteristics and lifestyle habits were independently associated with early fetal growth and that first-trimester fetal growth restriction was associated with an increased risk of adverse birth outcomes and growth acceleration in early childhood.

In **chapter 2.2**, we estimated the heritability for height and weight during fetal life and early childhood using regression models as proposed by Galton. Heritability estimates for height increased between second and third trimester from 13% to 28%. In the first month of postnatal life, height heritability estimates increased rapidly from 26% to 41%, followed by a gradual increase to 63% at 36 months. Heritability estimates for weight increased between 2nd and 3rd trimester from 17% to 27%, and gradually from 26% at birth to 42% at 36 months. Using the unique data from the Generation R Study, we demonstrated that heritability estimates of height and weight increase from second trimester to infancy.

In **chapter 2.3**, we validated a novel technique to examine visceral fat by means of ultrasound. For this we compared images of preperitoneal fat measured by computed tomography and by ultrasound in children. Then, in **chapter 2.4** we used this technique to assess the associations of fetal and postnatal growth characteristics with abdominal fat mass at the age of 2 years. We found that estimated fetal weight and birth weight were not associated with abdominal subcutaneous fat mass. Estimated fetal weight in second trimester of pregnancy was inversely associated with preperitoneal fat area. Weight gain from birth to the age of 2 years was positively associated with preperitoneal fat mass measures. Our results suggest that rapid growth rates during fetal life and infancy are associated with increased abdominal subcutaneous and preperitoneal fat mass in healthy children.

In **chapter 2.5**, we examined the associations of fetal growth characteristics with infant growth rates, and whether infant growth rates are associated with the risks of

overweight and obesity in early childhood. For this, we used derived variables during infancy: peak weight velocity (PWV), peak height velocity (PHV) and body mass index at adiposity peak (BMIAP). Estimated fetal weight in second trimester was positively associated with PWV and BMIAP. Subjects with a smaller weight increase between third trimester and birth had a higher PWV. Second trimester femur length was positively associated with PHV. Second and third trimester length increase was as negatively associated with PHV after birth. Compared to children in the lowest quintile of BMIAP, subjects in the highest quintile of BMIAP had a strongly increased risk of obesity at the age of 4 years. We concluded that there are strong associations between fetal and infant growth rates, and that infant growth patterns were also associated with the risk of obesity at 4 years of age.

In **chapter 3**, we explored a number of known obesity and type 2 diabetes genes in relation to early growth. In **chapter 3.1**, we examined the effect of *FTO* genotype, which is known to be associated with obesity in adulthood, on body composition at the age of 6 months using skinfold thickness measurements and Dual Energy X-ray Absorptiometry (DXA). No significant differences between *FTO* genotypes were found in weight, height, body mass index, skinfold thickness or any body composition measures (fat and lean mass) assessed by DXA. Thus, we concluded that there is no association between this *FTO* polymorphism and body composition at the age of 6 months.

In **chapter 3.2**, we explored the age-dependent association between this same *FTO* variant and BMI in children. For this, we meta-analyzed associations between *FTO* and BMI in samples aged from 2 weeks to 13 years from eight cohorts of European ancestry (N>19,000). We explored this association cross-sectionally and longitudinally by mixed effects models. Moreover, we examined the associations with infant adiposity peak (AP) and childhood adiposity rebound (AR). We identified a positive association between additional minor (A) alleles and BMI from 5.5 years onwards. In contrast, an inverse association was observed below age 2.5 years. In longitudinal analysis, the same variant was associated with lower BMI in infancy, higher BMI in childhood, and faster BMI growth rates in childhood. The A-allele was also associated with higher average BMI at AR and earlier AR. This study confirmed the expected association between variation at rs9939609 and BMI in childhood, but only after an inverse association between the same variant and BMI in infancy. The positive association between rs9939609 and BMI in childhood was manifest in both differential rates of BMI change and points of inflection in modelled BMI curves. The age-dependent nature of the association provides important evidence for a longitudinal gene effect.

In **chapter 3.3**, we examined the association between a polymorphism in *TCF7L2*, the strongest type 2 diabetes gene to date, and growth patterns from fetal life until infancy. Previously, an association was found between this genotype and birth weight, but the

authors of this study concluded that this effect was mostly an effect of maternal genotype rather than fetal genotype. This study was performed in the Generation R Study and a cohort of children born small-for-gestational-age (SGA cohort). No differences at birth were found in gestational age or size (head circumference, length, weight) between the fetal genotypes in either cohort. Fetal *TCF7L2* genotype was also not associated with any pre- or postnatal growth characteristic in either Generation R or the SGA cohort. Thus, we found no evidence for an association between fetal *TCF7L2* genotype and fetal and early postnatal growth.

In **chapter 3.4**, we examined whether the *PPARγ2* Ala12 allele influences growth in early life and whether this association is modified by breastfeeding. Growth rates in weight from second trimester of pregnancy to 18 months were higher amongst the Pro12Ala and Ala12Ala carriers compared to Pro12Pro carriers. We found an interaction between genotype and breastfeeding duration. In infants who were breastfed for at least four months, *PPARγ2* Pro12Ala was not associated with growth rate. When breastfeeding duration was shorter than two months or between two and four months, growth rate was higher in Ala12Ala subjects than Pro12Pro. We concluded that the *PPARγ2* Ala12 allele is associated with an increased growth rate in early life and that this effect may be influenced by breastfeeding duration.

Chapter 4 contains two studies regarding genome-wide association studies. In **chapter 4.1**, we set out to identify genetic variants associated with birth weight. For this, we meta-analyzed six genome-wide association (GWA) studies (N=10,623 Europeans from pregnancy/birth cohorts) and followed up two lead signals in thirteen replication studies (N=27,591). Rs900400 near *LEKR1* and *CCNL1*, and rs9883204 in *ADCY5* were robustly associated with birth weight. Correlated SNPs in *ADCY5* were recently implicated in regulation of glucose levels and type 2 diabetes, providing evidence that the well described association between lower birth weight and subsequent type 2 diabetes has a genetic component, distinct from the proposed role of programming by maternal nutrition. The impact on birth weight of these two SNPS is similar to that of a mother smoking 4-5 cigarettes per day in the third trimester of pregnancy.

In **chapter 4.2**, we followed-up on the two identified hits near *CCNL1/LEKR1* (rs900400) and in *ADCY5* (rs9883204) that are associated with birth weight. We examined the association of these variants with fetal growth characteristics in different trimesters, with a main interest in the timing of the associations and the affected body proportions. For this, we used data from two prospective cohort studies, the Generation R Study and the Raine Study from Australia. The C-allele of rs900400 was associated in second trimester with smaller fetal head circumference and femur length and in third trimester with smaller head circumference, abdominal circumference, femur length and estimated fetal weight. At birth, the C-allele of rs900400 was associated with all birth growth

characteristics and placental weight. The C-allele of rs9883204 was not associated with fetal growth in second trimester, but was associated with restriction of all growth characteristics, except head circumference, in third trimester and at birth. We found a strong association with lower placental weight amongst C-allele carriers of rs9883204. We concluded that rs900400 leads to slower fetal growth from early pregnancy onwards and a symmetric growth restriction, while rs9883204 leads to a slower fetal growth in third trimester and to asymmetric growth restriction.

In **chapter 5**, we reflect on the results of all studies included in this thesis in a broader context. Furthermore, we discuss methodological issues concerned in this field of epidemiological research and make suggestions for future research possibilities.

Samenvatting

Epidemiologische studies hebben een inverse relatie aangetoond tussen geboortegewicht en het risico op het ontwikkelen van type 2 diabetes. Deze relatie is behoorlijk sterk, hoewel de grootte van het effect wel iets kleiner is dan oorspronkelijk gedacht werd. Om het causale verband van deze associatie te verklaren zijn er twee hypotheses ontwikkeld: de 'Developmental Origins of Health and Disease hypothesis (DOHaD hypothese)', en de 'foetale insuline hypothese'. Het idee achter de DOHaD hypothese is dat suboptimale foetale groei tot foetale ondervoeding leidt. Deze ondervoeding kan vervolgens leiden tot permanente aanpassingen die gevolgen hebben voor de foetale groei, fysiologie en metabolisme. Dit fenomeen heet ook wel foetale programmering. Deze programmering kan op korte termijn de kans op overleving verhogen, maar kan tegelijkertijd ook negatieve lange termijn consequenties hebben voor de gezondheid. Het idee achter de 'foetale insuline hypothese' is dat veel voorkomende genetische varianten die gerelateerd zijn aan type 2 diabetes mogelijk, tenminste ten dele, de associatie tussen foetale groeivertraging en metabole veranderingen op volwassen leeftijd kunnen verklaren. Insuline is de belangrijkste groeifactor in utero en daarom zouden deze varianten zowel verminderde foetale groei, als een verhoogd risico op type 2 diabetes kunnen veroorzaken.

De meeste epidemiologische studies gebruiken geboortegewicht, een cross-sectionele meting, als een maat voor foetale groei. Echter, geboortegewicht is slechts het eindpunt van de foetale groei en het beginpunt van postnatale groei. Het zou goed kunnen dat er al foetale aanpassingen plaatsvinden voor de geboorte of zelfs in de eerste weken van de zwangerschap. Longitudinale studies hebben reeds aangetoond dat groeisnelheid ook van belang kan zijn voor de ontwikkeling van ziekte op latere leeftijd. Het gebruik van rechtstreeks gemeten foetale groeigegevens vanaf de vroege zwangerschap kan mogelijk ons inzicht in de timing van de foetale aanpassingen en de consequenties hiervan vergroten.

De drie belangrijkste doelen van dit proefschrift waren: 1) om te onderzoeken welke factoren van invloed zijn op de vroege groei en hoe de vroege groei zich relateert aan verdere groei, 2) om het effect op vroege groei te bestuderen van enkele bekende genetische varianten waarvan reeds bekend is dat ze geassocieerd zijn met obesitas en type 2 diabetes, en 3) om door middel van genoomwijde associatie studies nieuwe genetische varianten te identificeren die gerelateerd zijn aan vroege groei. Bij een belangrijk deel van het onderzoek voor dit proefschrift werd gebruikt gemaakt van data van de Generation R Studie, een onderzoek in Rotterdam waarbij bijna 10.000 kinderen vanaf de vroege zwangerschap gevolgd worden. Deze studie is opgezet om te onderzoeken welke factoren van invloed zijn op de groei en ontwikkeling van het kind. Een aantal van

de genetische studies in dit proefschrift is uitgevoerd in het kader van een samenwerkingsverband, bekend als het Early Growth Genetics (EGG) consortium.

In **hoofdstuk 2** bestuderen wij de relatie tussen vroege groei en lichaamssamenstelling en factoren die van invloed kunnen zijn op deze relatie. In **hoofdstuk 2.1** onderzoeken we welke risicofactoren van invloed zijn op de groei gedurende het eerste trimester van de zwangerschap. Hiervoor onderzochten we bij moeders met een bekende en betrouwbare menstruele cyclus de relatie tussen diverse maternale factoren en de foetale kruin-stuitlengte. Wij vonden dat maternale leeftijd, een lagere diastolische bloeddruk en een lager hematocriet geassocieerd waren met een grotere kruin-stuitlengte in het eerste trimester. Ook vonden we dat niet roken en het adequaat innemen van foliumzuur positief geassocieerd waren met de foetale groei in het eerste trimester. In vergelijking met normale foetale groei, hadden kinderen met foetale groeivertraging in het eerste trimester een 2 tot 3 keer zo hoog risico op vroeggeboorte en een laag geboortegewicht. Kruin-stuitlengte in het eerste trimester was bovendien negatief geassocieerd met een versnelde groei in de eerste twee levensjaren, een fenomeen dat gerelateerd is aan metabole en cardiovasculaire ziekte op volwassen leeftijd. Wij concludeerden dat maternale factoren en leefstijl invloed hebben op de foetale groei in het eerste trimester van de zwangerschap. Bovendien was vertraagde groei in het eerste trimester geassocieerd met slechte geboorte-uitkomsten en een versnelde groei postnataal.

In **hoofdstuk 2.2** geven we schattingen van de mate van erfelijkheid van lengte en gewicht tijdens de foetale en vroege postnatale groei middels het regressie model van Galton. Erfelijkheid van lengte nam tussen het tweede en derde trimester toe van 13% tot 28%. In de eerste maand postnataal, nam de erfelijkheid van lengte sterk toe van 26% tot 41%, gevolgd door een geleidelijke stijging tot 63% op de leeftijd van 36 maanden. Voor gewicht nam de erfelijkheid ook toe tussen het tweede en derde trimester van 17% tot 27%, en vervolgens geleidelijk van 26% bij de geboorte tot 42% op de leeftijd van 36 maanden. Aan de hand van de unieke data van Generation R, hebben wij kunnen aantonen dat de erfelijkheid van lengte en gewicht toeneemt vanaf het tweede trimester van de zwangerschap tot de peuterleeftijd.

In **hoofdstuk 2.3** valideren we een nieuwe techniek om visceraal vet te onderzoeken middels echografie. Hiervoor vergeleken we in kinderen het preperitoneaal vet dat door middel van CT (computed tomography) was gemeten met preperitoneaal vet dat middels echografie gemeten was. Hierna hebben we in **hoofdstuk 2.4** deze techniek gebruikt om de relatie tussen vroege groei en abdominale vet massa op de leeftijd van 2 jaar te bestuderen. Wij vonden dat zowel het geschatte foetale gewicht als het geboortegewicht niet gerelateerd waren aan de abdominale subcutane vet massa. Het geschatte foetale gewicht in het tweede trimester van de zwangerschap was invers geassocieerd met de preperitoneale vet massa. Gewichtstoename tussen de geboorte en

de leeftijd van 2 jaar was positief geassocieerd met de preperitoneale vet massa. Onze resultaten tonen aan dat versnelde groei zowel in utero als in de eerste jaren postnataal geassocieerd is met een hogere abdominale subcutane en preperitoneale vet massa in gezonde kinderen.

In **hoofdstuk 2.5** onderzoeken we de relatie tussen foetale groeipatronen en groeisnelheden in de vroege kinderleeftijd. Verder bekijken we of deze relatie mogelijk geassocieerd is met het risico op obesitas op kinderleeftijd. Hiervoor maakten we gebruik van de gecalculeerde variabelen peak weight velocity (PWV), peak height velocity (PHV) en body mass index bij adiposity peak (BMIAP). Het geschatte foetale gewicht in het tweede trimester was positief geassocieerd met PWV en BMIAP. Kinderen met een kleinere gewichtstoename tussen het derde trimester en geboorte hadden een hogere PWV. Femurlengte in het tweede trimester was positief geassocieerd met PHV. Lengtetoename gedurende het tweede en derde trimester was negatief geassocieerd met PHV. In vergelijking met kinderen in het laagste quintiel van BMIAP, hadden kinderen in het hoogste quintiel van BMIAP een sterk verhoogd risico op obesitas op de leeftijd van 4 jaar. Wij concludeerden dat er sterke relaties zijn tussen foetale en postnatale groeisnelheden en dat deze groeipatronen invloed hebben op het risico op obesitas op de leeftijd van 4 jaar.

In hoofdstuk 3 onderzoeken we een aantal obesitas en type 2 diabetes genen in relatie tot vroege groei. In **hoofdstuk 3.1** bekeken we of een variant van *FTO*, dat geassocieerd is met overgewicht bij volwassenen, gerelateerd is aan lichaamssamenstelling (vetplooimeting en Dual Energy X-ray Absorptiometry (DXA)) op de leeftijd van 6 maanden. Er waren geen significant verschillen in gewicht, lengte, body mass index, vetplooimetingen of andere maten van lichaamssamenstelling (gemeten door DXA) tussen de *FTO* genotypes. Dus wij concludeerden dat dit *FTO* polymorfisme niet gerelateerd is aan lichaamssamenstelling op de leeftijd van 6 maanden.

In **hoofdstuk 3.2** onderzoeken we of er een leeftijdsafhankelijk effect was van diezelfde *FTO* variant op BMI in kinderen. Hiervoor hebben we een meta-analyse uitgevoerd van de effecten van *FTO* op BMI van 8 studies, bestaande uit meer dan 19,000 kinderen met een leeftijd van 2 weken tot 13 jaar. Wij onderzochten de effecten zowel cross-sectioneel als longitudinaal. Bovendien analyseerden we de effecten op infant adiposity peak (AP) en childhood adiposity rebound (AR). Wij ontdekten een positieve associatie tussen het minor (A) allel en BMI vanaf 5.5 jaar. Daarentegen vonden we een negatieve associatie tussen dit allel en BMI voor de leeftijd van 2.5 jaar. In de longitudinale analyses vonden we dezelfde lagere BMI in de peuterleeftijd, gevolgd door een hogere BMI en hogere groeisnelheden op latere leeftijd. Het A-allel was ook geassocieerd met een hoger BMI op moment van AR en een jongere leeftijd van de AR. Concluderend bevestigt deze studie dat het effect van dit *FTO* genotype op BMI leeftijdsafhankelijk is.

In **hoofdstuk 3.3** onderzoeken we of een polymorfisme van het gen met het sterkste effect op type 2 diabetes, *TCF7L2*, gerelateerd is aan de foetale en vroeg postnatale groei. Uit eerder onderzoek is gebleken dat deze variant geassocieerd is met geboortegewicht, maar de auteurs van dit onderzoek concludeerden dat dit waarschijnlijk veroorzaakt werd door het maternale genotype in plaats van het foetale genotype. Deze studie werd uitgevoerd in de Generation R Studie en een cohort van kinderen die small-for-gestational-age waren geboren (SGA cohort). Er waren geen verschillen tussen de genotypes in zwangerschapsduur of geboortegewicht in beide cohorten. Het foetale *TCF7L2* genotype was ook niet geassocieerd met pre- of postnatale groei in beide cohorten. Concluderend vonden wij geen aanwijzingen dat dit foetale genotype effect heeft op de vroege groei.

In **hoofdstuk 3.4** onderzoeken we of het *PPARγ2* Ala12 allel vroege groei beïnvloedt en of deze associatie gemodificeerd wordt door borstvoeding. Groeisnelheid vanaf het tweede trimester van de zwangerschap tot een leeftijd van 18 maanden was hoger in de Pro12Ala and Ala12Ala dragers vergeleken met Pro12Pro dragers. We vonden een interactie tussen het foetale genotype and de duur van borstvoeding. In kinderen die tenminste 4 maanden borstvoeding hadden gekregen was er geen effect van het *PPARγ2* Pro12Ala allel. Maar wanneer er korter dan 4 maanden borstvoeding gegeven was, waren groeisnelheden hoger in de Ala12Ala kinderen. Wij concludeerden dat het *PPARγ2* Ala12 allel geassocieerd is met een verhoogde groeisnelheid en dat deze associatie gemodificeerd kan worden door de duur van borstvoeding.

Hoofdstuk 4 bevat twee studies die betrekking hebben tot genoomwijde associatie studies. In **hoofdstuk 4.1** was het doel om nieuwe genetische varianten te identificeren die gerelateerd zijn aan geboorte gewicht. Hiervoor hebben we zes genoomwijde associatie studies gemeta-analyseerd (N=10,623). Daarna werden de bovenste 2 signalen gerepliceerd in 13 andere studies (N=27,591). Rs900400 bij *LEKR1* en *CCNL1*, en rs9883204 in *ADCY5* waren sterk geassocieerd met geboortegewicht. Gecorreleerde SNPs in *ADCY5* zijn onlangs bevestigd een regulerende werking te hebben op glucose concentraties en type 2 diabetes. Hiermee is er nieuw bewijs geleverd dat de eerder omschreven relatie tussen laag geboortegewicht en type 2 diabetes mogelijk een genetische component bevat. Het effect van deze twee varianten is vergelijkbaar met het roken van 4-5 sigaretten per dag gedurende het derde trimester.

In **hoofdstuk 4.2** onderzoeken we of de twee geïndentificeerde hits bij *CCNL1/LEKR1* (rs900400) en in *ADCY5* (rs9883204), beiden gerelateerd aan geboortegewicht, ook geassocieerd zijn aan foetale groei tijdens de zwangerschap. De belangrijkste interesse was hoe deze SNPs relateerden aan foetale groei karakteristieken en wanneer tijdens de zwangerschap de effecten zichtbaar werden. Hiervoor maakten we gebruik van data van de Generation R Studie en de Raine Studie uit Australie. Het C allel van rs900400

was in het tweede trimester geassocieerd met een kleinere foetale hoofdomtrek en femurlengte en in het derde trimester met een kleinere hoofdomtrek, buikomtrek, femurlengte en het geschatte gewicht. Bij de geboorte was het C allel van rs900400 geassocieerd met alle geboortekarakteristieken en met placentagewicht. Het C allel van rs9883204 was niet geassocieerd met foetale groei in het tweede trimester, maar was wel geassocieerd met groeivertraging van alle groeikarakteristieken behoudens hoofdomtrek in het derde trimester en bij de geboorte. Wij vonden een sterke associatie met een lager placentagewicht bij dragers van het C allel van rs9883204. Wij concludeerden dat rs900400 leidt tot symmetrisch vertraagde groei vanaf de vroege zwangerschap en dat rs9883204 leidt tot asymmetrisch vertraagde groei, wat pas laat in de zwangerschap optreedt.

In **hoofdstuk 5**, bespreken we de resultaten van alle studies gepresenteerd in dit proefschrift in een bredere context. Bovendien bespreken we enkele methodologische onderwerpen die gepaard gaan met dergelijke studies en doen we aanbevelingen voor toekomstig onderzoek.

About the author

Dennis Owen Mook-Kanamori was born on June 28, 1979 in Enschede, the Netherlands. He is the second son of Byron Mook and Sakiko Kanamori. In 1997, he graduated from the Stedelijk Gymnasium in Arnhem, after which he spent two years at Swarthmore College, Pennsylvania, where his major was Classical Greek. In 1999, he returned to the Netherlands to start his medical education at the University of Leiden. As part of his medical training, he spent a year in Santiago de Chile (2003) at the Institute for Maternal and Child Research (IDIMI) with Prof F. Cassorla. During this year, he published his first papers in the field of pediatric epidemiology. In the final phase of his medical training, he worked at the Department of Pediatric Endocrinology (Professor J.M. Wit) at the Leiden University Medical Center. In November 2005, he graduated from Medical School and started working as a resident in pediatrics (ANIOS) at the Bronovo Hospital in the Hague. A year later he continued his residency (ANIOS) at the Sophia's Children's Hospital in Rotterdam. In 2007, he started his work as a Ph.D. student in the Growth and Development research group (Dr. V.W.V. Jaddoe and Prof. E.A.P. Steegers) at the Generation R Study, in collaboration with the Departments of Epidemiology (Prof. A. Hofman) and Pediatrics (Prof. A.J. van de Heijden) at the Erasmus Medical Center in Rotterdam. His research was primarily focused on the genetic and environmental factors that influence fetal and early postnatal growth. In the fall of 2010, he started his training to become a general practitioner at the Leiden University Medical Center, where he will also continue his research in the field of cardiovascular and metabolic epidemiology (Prof. W.J.J. Assendelft and Prof. J. Gussekloo). In May 2010, he married Marjonneke van Oosten. They are the proud and happy parents of Julien and live in Leiden.

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20. **Mook-Kanamori DO**, Steegers EAP, Aulchenko YS, Hofman A, Eilers PH, Jaddoe VWV. Heritability estimates of body size in fetal life and early childhood. The Generation R Study. *Submitted*
21. **Mook-Kanamori DO**, Durmus B, Sovio U, Hofman A, Jarvelin MR, Steegers EAP, Jaddoe VWV. The relationship between fetal and postnatal growth rates and the risk of obesity during infancy. *Submitted*
22. **Mook-Kanamori, DO**, Marsh JA, Steegers EAP, Warrington NM, Uitterlinden AG, van Duijn CM, Hofman A, Pennell CE, Palmer LJ, Jaddoe VWV. Associations of variants near *CCNL1/LEKR1* and in *ADCY5* with fetal growth patterns in different trimesters. *Submitted*
23. **Mook-Kanamori DO**, Sovio U, Warrington NM, Lawrence R, Briollais L, Palmer C, et al. Association between common variation at FTO locus and changes in body mass index from birth to adolescence: Longitudinal analysis of over 19,000 children of European ancestry. *Submitted*

PhD Portfolio

Summary of PhD training and teaching activities

Name PhD student:	Dennis Owen Mook-Kanamori
Erasmus MC Department:	Epidemiology and Pediatrics
Research School:	Netherlands Institute for Health Sciences
PhD period:	Jan 2007 – Nov 2010
Promotors:	Prof. Dr. A. Hofman and Prof. Dr. E.A.P. Steegers
Supervisor:	Dr. V.W.V. Jaddoe

1. PhD training

	Year	Workload (ECTS)
General research skills		
- Master's Degree Clinical Epidemiology, NIHES	2007-2009	
- Principles of Research in Medicine	2007	0.7
- Clinical Decision Analysis	2007	0.7
- Methods of Public Health Research	2007	0.7
- Classical Methods for Data-Analysis	2007	5.7
- Modern Statistical Methods	2007	4.3
- Introduction to Decision-making in Medicine	2007	0.7
- Topics of Aging Research	2007	0.7
- Topics of Health and Diseases in the Elderly	2007	0.7
- Cohort Studies	2008	0.7
- Study Design	2008	4.3
- Clinical Epidemiology	2008	5.7
- Methodological Topics in Epidemiological Research	2008	1.4
- History of Epidemiological Ideas	2008	0.7
- Conceptual Foundation of Epidemiological Study Design	2009	0.7
- Health Economics	2009	0.7
- Principles of Genetic Epidemiology	2009	0.7
- Primary and Secondary Prevention Research	2009	0.7
In-depth courses		
- SNP and human disease, MolMed	2007	1.4
- Advances in Population-based Studies of Complex Genetic Disorders	2008	1.4
- Repeated Measurements in Clinical Studies	2009	1.9
- Missing values in Clinical Research	2009	0.9
- Analysis of Time-varying Exposures	2009	0.9
- Advanced Analysis of Prognosis Studies	2009	0.9
Symposia		
- New Concept of Fetal Growth and Development	2007	0.3
- Prenatal and Early Postnatal Brain Development	2008	0.3
- 40 years Epidemiology at ErasmusMC	2009	0.3

(Inter)national conferences and presentations		
- Integrale Jeugdzorg. Arnhem, The Netherlands. Oral presentation: The Generation R Study	2008	0.2
- European Society for Paediatric Research. Nice, France. Poster presentation: PPARγ-2 Pro12Ala polymorphism and growth in fetal and early postnatal life.	2008	0.7
- King Faisal Specialist Hospital and Research Center. Riyadh, Saudi Arabia. Oral presentation: The Generation R Study	2009	0.2
- University of Bristol. Bristol, U.K. Oral presentation: The Generation R Study	2009	0.2
- European Society for Paediatric Research. Hamburg, Germany. Oral presentations: Brain-Derived Neurotrophic Factor (BDNF) gene polymorphism, attention problems and weight gain in childhood; and Type 2 Diabetes gene HHEX affects growth in early life.	2009	1.4
- TNO Research. Zeist, The Netherlands: Oral presentation: Risk factors and consequences of first trimester growth retardation	2009	0.2
- The Institute for Mother and Child Research (IDIMI). Santiago de Chile, Chile. Oral presentation: The Generation R Study	2009	0.2
- DOHaD meeting. Santiago de Chile, Chile. Oral presentation: Risk factors and consequences of first trimester growth retardation	2009	1.4
- Bataafsch Genootschap. Rotterdam, The Netherlands. Oral Presentation: The Generation R Study	2010	0.2
- The Pasteur Institute. Lille, France. Oral presentation: The Generation R Study	2010	0.2
- Birth Cohort Meeting, Oslo, Normay. Oral presentation: Risk factors and consequences of first trimester growth restriction	2010	0.2
Reviewing papers		
- Review papers for various international journals (8 times) European Journal of Epidemiology (3 times) European Journal of Haematology (1 time) American Journal of Obstetrics and Gynecology (1 time) Pharmacogenetics and Genomics (1 time) Pediatric Research (1 time) Diabetes Research and Clinical Practice (1 time)	2008-2010	1.6

2. Teaching

	Year	**Workload (ECTS)**
Supervising Master's theses		
- Supervisor Vera van Houten, Clinical Epidemiology, NIHES. Thesis Topic: A variant of the IGF-I gene is associated with blood pressure but not with left heart dimensions at the age of 2 years.	2007	2.0
- Supervisor Loes Hollestein, Biomedical Research, University Leiden. Thesis Topic: Abdominal fat in children measured by ultrasound and computed tomography.	2008	3.0
- Supervisor Büşra Durmuş, Clinical Epidemiology, NIHES. Thesis Topic: Growth in fetal life and infancy is associated with abdominal adiposity at the age of 2 years.	2009	2.0

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